The Function and Regulation of Starch Degradation during Plant Responses to Abiotic Stress

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ABSTRACT

Plants are sessile organisms and thus cannot evade adverse environmental conditions like cold, heat, flooding, drought, or soil contamination with salt or heavy metal ions. Such abiotic stresses impair plant growth, reduce the number of viable seeds a plant produces and in extreme cases lead to death before the plant can complete its life cycle. Abiotic stresses are also a major factor limiting the productive potential of crop plants. It is estimated that up to 80% of the potential yield is lost due to abiotic stress. Water stress is the most common and devastating abiotic stress, accounting for over 40% of the total yield loss. Global water shortage is expected to worsen due to increasing population and climate change. The Intergovernmental Panel on Climate Change (IPCC) has concluded that the increasing greenhouse gas concentrations are likely to lead to the general drying of the subtropics by the end of this century, creating widespread drought stress in agriculture.

On a cellular level, water stress impairs plant growth by reducing turgor of expanding cells and by changing the properties of the cell wall. Photosynthesis declines due to reduced CO_2 uptake and reactive oxygen species (ROS) are produced by absorption of excess light, damaging proteins, membranes and DNA. Furthermore, lack of water eventually denatures proteins and disrupts membranes, leading to cell death. Plants have evolved a variety of responses to minimize the impact of abiotic stress, preventing cellular damage, allowing for continued water uptake and growth under adverse conditions. Accumulation of so called “compatible solutes” (e.g. sugars, polyols, proline and betaine) plays a key role. On the one hand, they lower the internal water potential allowing continued uptake of water; on the other hand, they directly protect proteins and membranes from damage allowing them to remain functional under water limiting conditions. Starch is an important carbohydrate storage compound, and there is substantial evidence that the rapid mobilization of starch in the leaves during stress contributes to stress tolerance. Sugars released from starch could be used for the synthesis of compatible solutes and to sustain the central metabolism when photosynthesis is impaired. However, it is not known which enzymes are responsible for this stress-induced starch degradation, nor how their activity is regulated.

To answer these questions we examined stress-induced changes of carbohydrate metabolism and distribution of photoassimilates in *Arabidopsis thaliana*. Our

experiments revealed that starch is rapidly degraded upon osmotic stress in leaves of Arabidopsis. We further identified two specialised starch degrading enzymes, α-amylase 3 (AMY3) and β-amylase 1 (BAM1), which are not required for nocturnal starch degradation, but work synergistically to degrade starch during stress. The primary breakdown product of starch, maltose, is subsequently further metabolised to other sugars, mainly sucrose. Sucrose is exported to the roots where it promotes root elongation and water uptake, counteracting the effects of stress. The *amy3bam1* mutant is unable to degrade starch in response to osmotic stress and is more sensitive to stress, showing reduced root growth and water uptake compared to wild-type plants. We also showed that *BAM1* and *AMY3* expression is induced by osmotic stress in an abscisic acid (ABA)-dependent manner, and the promoters of both genes contained ABA responsive elements (ABRE). Additionally, mutants impaired in ABA synthesis or signalling cannot degrade starch during osmotic stress, whereas exogenous ABA triggers starch degradation even in the absence of stress.

We further showed that the mechanism we identified in Arabidopsis is likely conserved among plants. An exhaustive analysis of β-amylase genes from over 100 plant species revealed that BAM1 is conserved amongst seed plants, as are the ABREs, suggesting that *BAM1* orthologs are likely induced by ABA and stress. This conservation is remarkable, as our phylogenetic analysis revealed more substantial divergences in other β-amylases.

A survey of the existing literature further strengthened the hypothesis that induction of starch degradation is a general stress response in the plant kingdom. The majority of considered studies found a significant reduction of starch content during abiotic stress, not only in higher plants, but also basal land plants and even algae.

We also investigated a potential role of posttranslational regulation of BAM1 during stress, which manifested as a change in apparent molecular weight. However, in contrast to clear evidence of transcriptional regulation of BAM1 activity during stress, we could not conclusively identify the nature and the role of post-translational regulation.

Taken together, the data presented in this thesis extend our knowledge of stress-induced changes of starch and carbohydrate metabolism. We provide novel insights into the regulation and function of stress-induced starch degradation and show that it is a conserved response to stress in the plant kingdom. Due to the importance of compatible solutes (such as carbohydrates) for drought tolerance, the starch and its metabolism are potential targets for engineering more drought tolerant crop plants.

ZUSAMMENFASSUNG

Pflanzen sind im wahrsten Sinne des Wortes angewurzelt, und können deshalb widrigen Umweltbedingungen wie Frost, Hitze, Dürre oder mit Schwermetall belasteten Böden nicht ausweichen. Solche abiotischen Stressfaktoren verringern nicht nur das Wachstum der Pflanze, sondern auch die Anzahl Samen, und führen schlimmstenfalls zum Tod der Pflanze. Diese Auswirkungen führen auch in der Landwirtschaft zu erheblichen Ernteeinbussen, und es wird geschätzt dass über 80% des theoretisch möglichen Ertrages durch abiotischen Stress vernichtet wird. Wassermangel ist der am weitesten verbreitete Stress und etwa 40% der Ernteeinbussen gehen bereits heute auf Trockenheit zurück. Weltweit wird sich der Wassermangel aufgrund des Bevölkerungswachstum und des Klimawandels in Zukunft noch verschärfen. Der Weltklimarat (IPCC) kam zum Schluss, dass die erhöhten Treibhausgaskonzentrationen noch in diesem Jahrhundert zu einem deutlich trockeneren Klima in den subtropischen Gebieten führen werden, mit verheerenden Folgen für die dortige Landwirtschaft.

Zum einen behindert Wassermangel direkt das Wachstum der Pflanze indem der Turgordruck einzelner Zellen sinkt und die Zellwände verhärtet. Andererseits schliessen die Pflanzen als Reaktion auf Wassermangel die Spaltöffnungen, wodurch zwar weniger Wasser verloren geht aber auch weniger CO_2 aufgenommen werden kann. Dies vermindert die Photosyntheseleistung und die nicht benötigte Lichtenergie führt zur Bildung von freien Radikalen, welche die Zellmembran, Proteine und DNS beschädigen. Dauert die Trockenheit an, denaturiert sie letztlich die Proteine und Membranen was zum Tod der Pflanzenzellen führt. Im Laufe der Evolution haben Pflanzen verschiedene Strategien entwickelt um die von Trockenheit verursachten Schäden zu vermeiden, und auch unter widrigen Umweltbedingungen zu wachsen und gedeihen. Eine zentrale Rolle nehmen dabei die sogenannten „kompatiblen Solute“ wie Zucker, Polyole, Prolin und verschiedene Betaine ein. Einerseits verringern diese Stoffe das Wasserpotential der Zelle was es ermöglicht auch während Trockenheit Wasser aufzunehmen, andererseits stabilisieren sie Proteine und Membranen welche so ihre Funktion auch unter Wassermangel ausüben können. Stärke ist ein wichtiger Zuckerspeicher und es verschiedene Studien zeigten, dass der schnelle Abbau von Stärke die Stressresistenz von Pflanzen erhöht. Allerdings war es bis jetzt nicht bekannt welche Enzyme für den Stress-bedingten Abbau benötigt werden, noch wie ihre Aktivität kontrolliert wird.

Um diese Fragen zu beantworten untersuchten wir die von Wassermangel ausgelösten Änderungen des Stoffwechsels in *Arabidopsis thaliana*. Unsere Untersuchungen zeigten, dass Wassermangel einen raschen Stärkeabbau in den Blättern von Arabidopsis auslöst. Des Weiteren identifizierten wir zwei Enzyme – α-Amylase 3 (AMY3) und β-Amylase 1 (BAM1) – welche Stärke während Wassermangel abbauen nicht aber während der Nacht. Die von der Stärke abgespalteten Zucker, vor allem Maltose, werden weiter verstoffwechselt, hauptsächlich zu Saccharose. Saccharose wird dann in die Wurzeln exportiert wo es das Wachstum und die Wasseraufnahme fördert, und so den Wassermangel reduziert. Die *amy3bam1* Mutante ist nicht mehr in der Lage Stärke während dem Wassermangel abzubauen und ist anfälliger für Trockenheit, da ihre Wurzeln weniger Wasser aufnehmen können und in Zeiten des Wassermangels langsamer wachsen. Ausserdem konnten wir zeigen, dass die durch Wassermangel ausgelöste erhöhte Transkription von BAM1 und AMY3 von dem bekannten Stresshormon Abszisinsäure (ABA) abhängt. In den Promotoren beider Gene finden sich sogenannte ABA responsive elements (ABRE) welche benötigt werden damit ABA die Transkription eines Genes steigern kann. In Mutanten welche entweder kein ABA herstellen können oder nicht in der Lage sind es wahrzunehmen löst Wassermangel keinen Stärkeabbau aus, während von aussen zugeführtes ABA auch bei genügender Wasserversorgung Stärkeabbau auslösen konnte.

Die Untersuchung der Genome anderer Pflanzen deutet darauf hin, dass der von uns in Arabidopsis gefundene Mechanismus auch in anderen Pflanzen vorkommt. Die Analyse der β-Amylasen von über 100 Pflanzenarten zeigte, dass BAM1 in allen vorkommt. Auch die ABRE wurden in den Promotoren anderer Pflanzen gefunden, so dass davon ausgegangen werden kann, dass auch diese Gene von ABA induziert werden können. Diese Erhaltung ist insbesondre deshalb bemerkenswert weil wir in anderen β-Amylasen wesentliche Unterschiede feststellen konnten.

Die Theorie dass Stärkeabbau eine in allen Pflanzen vorkommende Stressantwort ist, wird auch von einer umfassenden Analyse der existierenden Literatur gestützt. Die überwiegende Mehrheit der Studien kam zum Schluss, dass verschieden abiotische Stressfaktoren Stärkeabbau auslösen, nicht nur in höheren Pflanzen sondern auch in Moosen und Algen.

Zuletzt untersuchten wir auch ob post-translationale Modifikationen die Aktivität von BAM1 während dem Wasserstress beeinflussen. Allerdings konnten wir im Gegensatz zu der klaren Änderung der Genexpression keine eindeutigen post-translationalen Änderungen nachweisen.

Zusammengenommen erweitern die in dieser Arbeit dargestellten Ergebnisse unser Wissen über die von Stress verursachten Veränderungen des Stärke- und Zuckerstoffwechsels der Pflanzen. Wir konnten nicht nur neue Erkenntnisse über die Regulation und Funktion des Stärkeabbaus während abiotischem Stress finden, sondern zeigten auch dass diese Mechanismen in allen Pflanzen vorkommen. Die rasche Synthese von kompatiblen Soluten – wie die von der Stärke abgespaltenen Zucker – ist für die Trockenresistenz von Pflanzen unabdingbar, daher ist es möglich das gezielte Eingriffe in den Stärkestoffwechsel die Trockenresistenz von wichtigen Nutzpflanzen erhöht.

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INTRODUCTION

Context of this thesis

The world population is increasing apace and is expected to exceed 10 billion by the end of this century (Gerland et al., 2014), requiring a concomitant increase in food production. In the past, agricultural production managed to keep up with the population growth. In the 18th and 19th century, the industrial revolution and mechanisation of agriculture delivered the necessary increase, as did the "Green Revolution" in the 20th century. However, these increases were just about sufficient to keep up with the increasing demands, leaving no or only little surpluses. Thus, despite the enormous increases in food production, 800 million people – 10% of the world population – are still undernourished and many more suffer from micronutrient deficiencies (Food and Agricultural Organisation of the United Nations, 2015). Further increases of food production will be necessary over the course of the 21th century to keep up with the projected population growth. Unfortunately, previous increases of agricultural output relied mainly on the use of non-renewable resources, such as fertilizers and fuels, as well as the overexploitation of resources like land and water that would be in principle renewable. This intensification of agriculture cannot be sustained indefinitely and will eventually lead to permanent loss of arable land through desertification. Furthermore, climate change is expected to result in warmer temperatures, changes to rainfall patterns, and more frequent and severe extreme weather events, all of which will negatively affect food production (Wheeler and Braun, 2013). For example, the expansion of the tropics will result in a drying of today's sub-tropical regions devastating local agriculture (Seidel et al., 2008; Shang et al., 2007).

To increase or even maintain food productivity will be essential to develop crop plants well adapted to the drier climatic conditions, which can withstand abiotic stress imposed by drought. This will allow high biomass production even under unfavourable growth conditions. A deeper understanding of plant physiology and molecular mechanisms of drought tolerance will be invaluable to develop such plants. Detailed knowledge of these mechanisms will reveal new targets for modern breeding approaches. These will include both the exploitation of the natural genetic diversity of today's most important crop plants and their wild relatives, but also the creation of genetically modified varieties, ultimately leading to an improved combination of traits.

Abiotic stress

What is stress?

The stress concept was pioneered by Hans Selye, who observed that exposure of rats to a broad variety of harmful stimuli – physical injury, overexertion, cold and different poisons – results in a similar response, named “stress”. As Selye himself summarised, a key insight of his work was that “There exist stressor-specific responses and non-specific general responses.” (Selye, 1936, 1950). Selye also recognised that stress is a dynamic response. A first stage, which he named “general alarm reaction”, is dominated by stress-induced damage and impairment of organ function. In a second stage, named “general adaptation syndrome”, various defence reaction allow the organism to restore normal functionality in the presence of stress (Selye, 1936). Although Selye focused his research on animals, the stress concept was applied to other organisms, including plants.

In plant biology Selye’s stress concept was merged with the resistance concept of Levitt, which defined stress as “Any environmental factor potentially unfavourable to living organisms”, while the responses to stress were named “strain” (Levitt, 1980). Levitt focused mostly on stress resistance mechanisms which he subdivided into escape, tolerance and avoidance. The current stress model in plant biology represents a synthesis of these two models (Larcher, 1987), and was reviewed by (Lichtenthaler, 1998), who divided the plant stress response into four distinct phases (Figure 1):

I: **Alarm phase** (Beginning of stress) is characterised by the negative impacts of the stressor, redox-imbalance, impairment of metabolic processes and growth. In extreme cases the plant may succumb to stress during this phase.

II: **Restitution phase** (continuing stress) is characterised by successful adaptation and resistance to stress. This allows the plant to repair the damage caused during the first phase and to resume growth. Further responses depend on the severity of the stress. In the case of moderate stress, the plant may persist in this stage for over a century (Lichtenthaler, 1998). In more severe cases, the stress response will progress to the third phase.

III: **Exhaustion phase** (severe long term stress) is characterised by a gradual exhaustion of the plant defences leading to cell damage, premature senescence, and eventually death of the plant. However, in nature stresses are often temporary and upon timely removal of the stresses plant will enter the last phase.

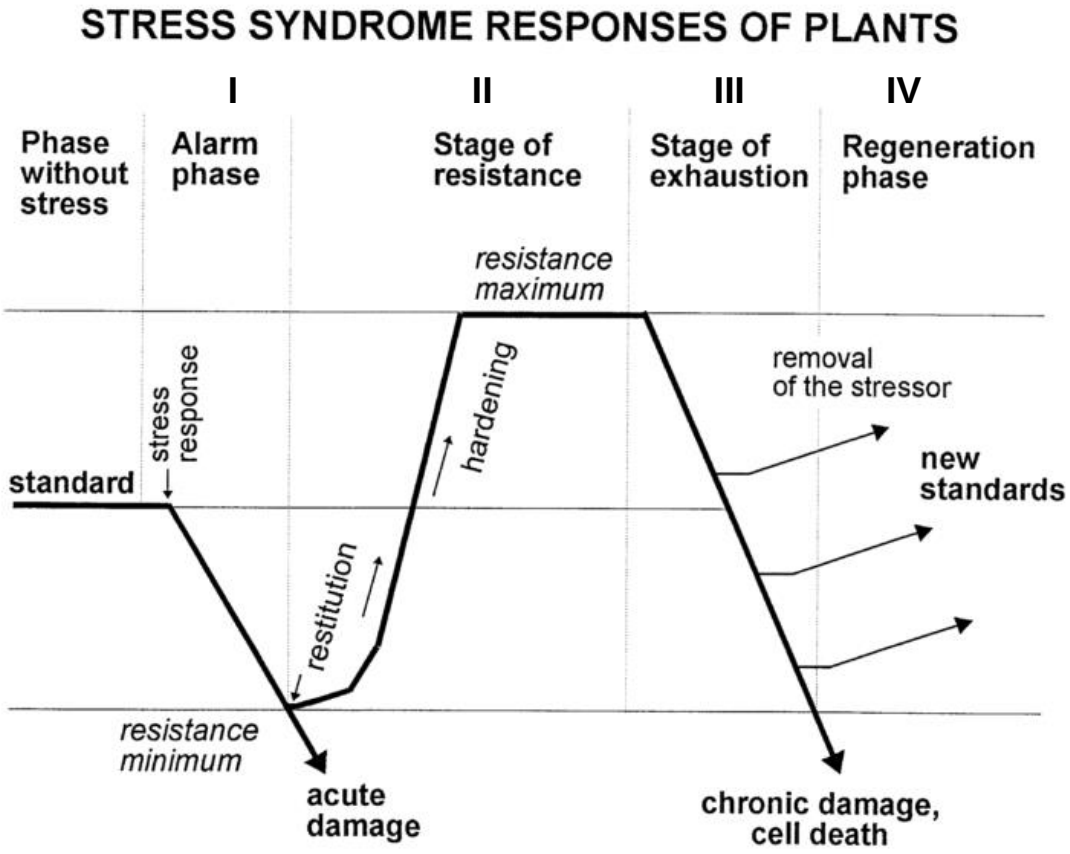

Figure 1: General stress concept describing the different phases of stress responses. Adapted from (Lichtenthaler, 1998).

IV: **Regeneration phase** (after stress) is characterised by regeneration, damage repair and eventually the return to normal function.

It is important to note that in this model, stress does not necessarily lead to damages in a plant. If intensity and duration of stress are low and short enough, plants will remain in the stage of resistance, ensuring continued health. However, the cost of stress responses often leads to lower productivity and ultimately reduced fitness (Krasensky and Jonak, 2012).

In nature, plants encounter many different types of stresses, which are commonly subdivided into biotic and abiotic stresses (Lichtenthaler, 1998; Orcutt and Nilsen, 2000). Biotic stress is caused by interaction with other living organisms, such as pathogens, parasitic weeds (Musselman, 1980), herbivores, and competition with other plants. In contrast, abiotic stress is caused by adverse environmental conditions. Broadly speaking, the lack or excess of virtually any environmental factor can constitute stress, although the thresholds of what constitutes stress are of course different for different species or even different races and ecotypes (Niinemets, 2010).

Water stress

Water is essential for every form of life, and the loss of water invariably leads to the cessation of all biological activities, and in most cases the death of the organism – although some species, such as resurrection plants, can restore biological activities upon rehydration (Farrant, 2000). Thus, it should be no surprise that water scarcity is one of the major limitation of plant productivity (Boyer, 1982). The concept of water potential is useful to describe water stress as it allows to quantify the availability of water for plants. The water potential (Ψ_w) is a measure of the free energy of water. Pure water or air at 100% relative humidity have a water potential of 0; as the water availability decreases, so does the water potential – i.e. it becomes negative. A system completely devoid of water has an infinitely negative water potential ($-\infty$). The water potential of a given system can be calculated as the sum of several independent potentials, the osmotic potential (Ψ_S), the pressure potential (Ψ_p), the matrix potential (Ψ_m), and the gravimetric potential (Ψ_g) (Boyer and Kramer, 1995):

$$\Psi_w = \Psi_s + \Psi_p + \Psi_m + \Psi_g$$

The gravimetric potential describes the effect of height differences. This is relevant for tall plants (e.g. trees), but negligible for herbaceous plants. The matrix potential describes the interaction of water with the soil and depends on the type of soil.

The two most important contributions to the internal water potential of plants are the osmotic potential and the pressure potential (Verslues et al., 2006). The pressure potential is derived from the turgor of plant cells; higher pressure results in a higher (i.e. more positive) the pressure potential. The osmotic potential depends on the concentration of dissolved solutes, and is lower (i.e. more negative) at higher concentrations. Its exact value can be calculated using the van't Hoff equation:

$$\Psi_s = -c \times R \times T$$

Where c is the concentration of the solutes, R the universal gas constant and T the absolute temperature.

As water moves only from high potential toward low potentials, plants can only take up water from the soil as long as their roots are at a lower water potential than the soil. If the water potential of the root – typically around -0.5 MPa (Aston and Lawlor, 1979) – exceeds the soil water potential the plant experiences severe water stress. Consequently, water stress can be caused by any stress that lowers the water potential of the soil, such as, drought, salinity, cold, or osmotic stress.

Strictly speaking, osmotic stress can refer either to hypo-osmotic stress (the extracellular space is at a higher water potential than the cytosol) or hyper-osmotic stress (the extracellular space is at a lower water potential than the cytosol). Because

the plant cell wall generally offers robust protection against hypo-osmotic stress, in plant sciences osmotic stress is often used to refer to hyper-osmotic stress only (Zhu, 2016). Osmotic stress lowers the solute potential of the soil, preventing the roots from absorbing water. Indeed, osmotic stress causes efflux of water from the cytosol, leading to loss of turgor and the detachment of the plasma membrane from the cell wall, an event which is called “plasmolysis”. In nature, osmotic stress usually occurs as part of salinity stress (see below). In laboratory research, osmotic stress is often used as a model system to simulate the effects of drought. By adding solutes like mannitol, sorbitol, and polyethylene glycol (PEG) to the growth media, the water potential can be lowered to a defined value, simulating what happens in drying soil in a controlled manner. For example, the water potential of a 300 mM solution, which is used for the experiments presented in chapter 1, reduces the water potential at room temperature to -0.73 MPa, causing severe stress. Conversely, the water potential of a 150 mM solution, which is used for the experiments presented in Chapter 2, reduces the water potential to only -0.37 MPa, imposing a mild stress on the plants (Fig. 2).

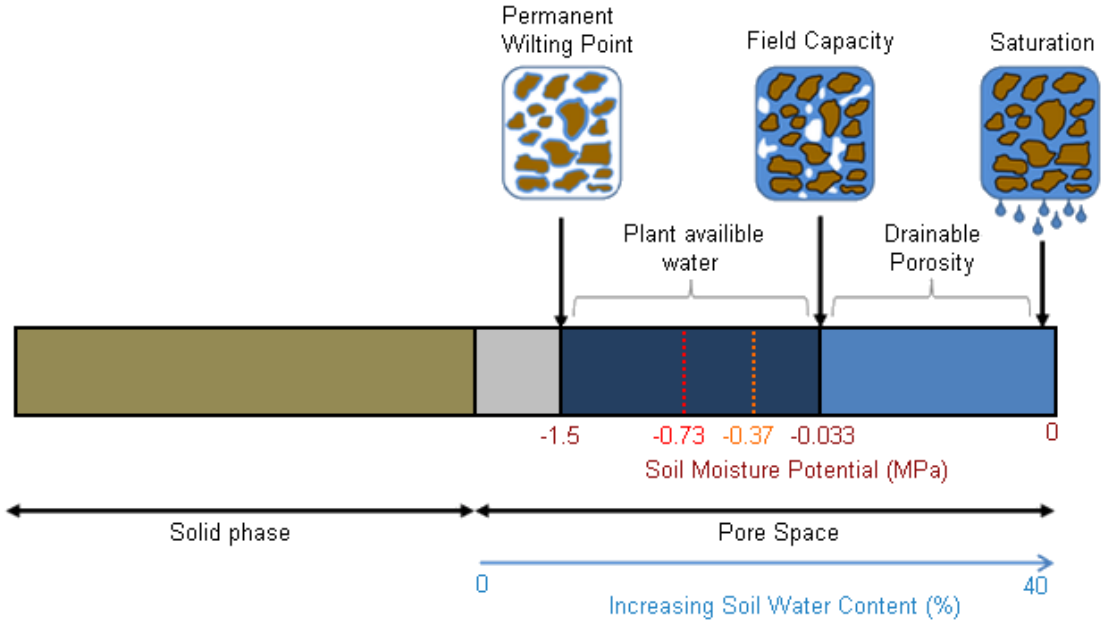

Figure 2: Water content and water potential at saturation, field capacity and permanent wilting point. The difference in water content between field capacity and permanent wilting point is plant available water. Drainable porosity is the amount of water that drains from macropores by gravity between saturation to field capacity typically representing three days of drainage in the field. The water potential of the solutions used in chapter 1 and 2 are indicated in red and orange respectively. Figure adapted from (O’Geen, 2012)

During drought stress, the soil dries out, lowering the matrix potential, and consequently the water potential. If the soil water potential drops below -1.5 MPa plants are unable to take up water and will begin to wilt, hence this water potential is also named permanent wilting point (Fig. 2) (O'Geen, 2012). As air cannot permeate the cell wall, the loss of water will eventually lead to the collapse of the cell walls (cytorrhysis).

In addition to the ion-toxicity, salt stress also has an osmotic component which dominates the first phase of salt stress and causes water stress (Munns and Tester, 2008). Only at later stages shoot ion concentrations reach levels that are toxic to the plant, leading to accelerated senescence.

During freezing, ice crystals form, and this crystallisation lowers the water potential, leading to the dehydration of cells (Beck et al., 2007). Thus, cold stress also induces water stress.

Perception of water stress

As water stress elicits – in addition to common responses – specific changes to gene expression, metabolism and physiology, it stands to reason that plant cells can perceive water stress directly and not just the indirect effects on cellular functions. However, the search for plant osmosensors has been difficult, and despite much effort, has so far yielded only few candidates. A possible explanation is the functional redundancy of genes encoding sensor proteins so that the loss of a single gene does not result in a measurable phenotype – a problem that has already greatly hindered the elucidation of ABA signalling (Park et al., 2009). It is also possible that a sensor is essential for plant survival and its loss is lethal to the plant. Furthermore, proving that a protein responds directly to a physical signal (such as changes in water potential) is experimentally challenging. The situation is further complicated by positive feedback loops where a stimulus can be both a primary signal and a secondary messenger. For example, while Ca^{2+}-spikes can be triggered directly by stress, the phytohormone ABA also increases the intracellular Ca^{2+} concentration (Knight, 1999; Webb et al., 2001; Zhu, 2016).

Arabidopsis histidine kinase 1 (AHK1) has been proposed as an osmosensor based on its homology to the yeast osmosensors synthetic lethal of N-end rule 1 (SNL1) and SH3-Domain osmosensor 1 (SHO1), its ability to complement the *sln1/sno1* double mutant (Urao et al., 1999), and the increased drought sensitivity of the *ahk1* mutant (Wohlbach et al., 2008). However, subsequent work revealed that several other plant histidine kinases, which had been shown to be cytokine receptors *in planta*, could also complement the *sln1/sno1* yeast mutants (Tran et al., 2007), indicating that

complementation assays cannot establish the *in planta* function of AHK1. Careful analysis of *ahk1* mutants also revealed that its enhanced drought sensitivity was linked to an increased stomatal density, while water stress responses were not impaired (Kumar et al., 2013).

More recently, reduced hyperosmolaltiy-induced calcium increase 1 (OSCA1) was identified as a putative sensor of osmotic stress in Arabidopsis (Yuan et al., 2014). Cytosolic Ca^{2+} is an important secondary messenger and its concentration rapidly increases in response to osmotic stress agents (including salt), extreme temperature, heavy metals, oxidative stress and the phytohormone ABA (Zhu, 2016). This Ca^{2+}-spike was strongly reduced in *osca1* mutants specifically in response to osmotic stress. Furthermore, stomatal closure and root growth during osmotic stress were impaired in *osca1* mutants. It is unclear how OSCA1 perceives osmotic stress. It is possible that OSCA1 is a mechano-sensing channel, reacting not to changes in water potential itself, but to changes in turgor and membrane tension. Multiple families of mechano-sensing proteins – such as DEG/ENaC, K_{2P}, MscS-like, Piezo, and TRP – have been identified in eukaryotes (Árnadóttir and Chalfie, 2010). Two families, MscS-like and Piezo, are also found in Arabidopsis (Hedrich, 2012), but OSCA1 does not belong to either of these protein families. Further work will be necessary to unambiguously establish the role of OSCA1 in perception of stress.

Passing the signal on

In contrast to the sparse knowledge about the primary receptors of osmotic stress, significantly more is known about subsequent downstream signalling events (Fig. 4) (reviewed by Zhu, 2016). The signalling cascades are commonly divided into an ABA-dependent pathway and an ABA-independent pathway (Yoshida et al., 2014b), although there are of course links between the two pathways. The signal transduction of ABA will be discussed in detail in the second chapter of the introduction, and represents a key part of water stress signalling. However, additional pathways are involved in signal transduction.

In many signal transduction pathways, the phosphorylation and dephosphorylation of proteins mediated by kinases and phosphatases, respectively, is an essential and effective mechanism for signal relay. Abiotic stress response is no exception and there are several kinase families involved in stress-mediated signalling.

Abiotic stresses, including osmotic stress, trigger an immediate increase in intracellular calcium concentration (Dodd et al., 2010). This calcium-spike is decoded by calcium binding proteins, such as calcineuring B-like (CBL) and calmodulin-like (CML), as well as kinases like calcium dependent protein kinase (CPK) calcium or calmodulin-

dependent protein kinase (CCaMK). Some CPKs are positive regulators of ABA signalling, but are not themselves stimulated by ABA (Lu et al., 2013), while others are induced by ABA (Geiger et al., 2010).

Mitogen-activated protein (MAP) kinases constitute a highly conserved signalling module among eukaryote organisms including plants (Taj et al., 2010). As their name suggest they, control cell division. However, MAP kinases are also involved in many other processes such as cell differentiation, and organ development, but also responses to abiotic stresses such as salinity, or drought (Sinha et al., 2011). A MAP kinase cascade consist of at least three kinases which phosphorylate and activate each other: a MAP kinase kinase kinase (MAPKKK, MEKK), a MAP kinase kinase (MAPKK, MEK) and a MAP kinase (MAPK). MAPKs phosphorylate a broad range of substrates reprogramming transcription and modulating the activity of existing proteins (Fig. 3). The relationship between ABA and MAP kinases is complex and was recently reviewed by (de Zelicourt et al., 2016). While the activation of some MAPK cascades is independent of ABA (Jonak et al., 1996), others appear to mediate the effects of ABA (Jiang and Song, 2008).

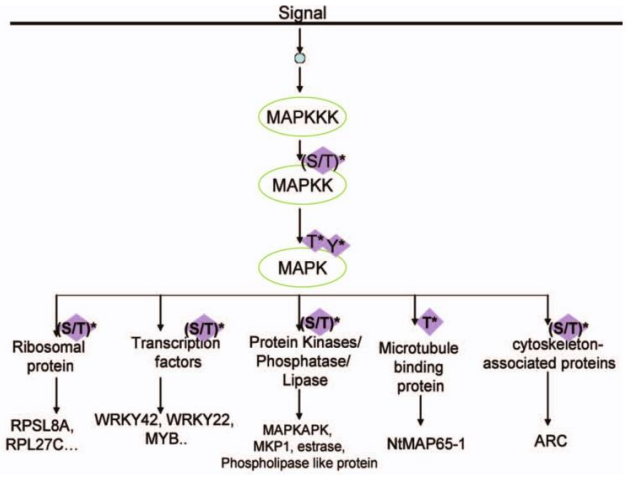

Figure 3: A generic MAP kinase cascade consists of a series of phosphorylation events culminating in in the activation of diverse targets eliciting a specific response. Adapted from (Taj et al., 2010)

Another group of kinases involved in stress signalling are the Sucrose non-fermenting Related Kinases (SnRK). Members of the SnRK family are found in all eukaryotes and generally serve as metabolic sensors and energy gauges (Hardie et al., 1998). In plants, SnRKs are divided into three families, SnRK1 to 3, and are thought to link carbon metabolism and stress signalling (Coello et al., 2011). SnRKs from family 1 (SnRK1), which are most similar to fungal and metazoan SnRKs, control carbon and energy metabolism, both through changes in transcription and post-translational regulation of enzyme activity (Halford and Hey, 2009). The loss of SnRK1 activity causes numerous defects in carbon allocation, impairing for example the nocturnal remobilisation of starch (Thelander et al., 2004). The more divergent SnRK2 and SnRK3 are involved in signal transduction of osmotic and salt stress, respectively (Coello et al., 2011; Halford and Hey, 2009). Of the ten Arabidopsis SnRK2s, all but

one are activated by osmotic stress (Boudsocq et al., 2004), and the decuple mutant disrupted in all SnRK2 genes is highly sensitive to osmotic stress. Interestingly, the decuple mutant does not accumulate ABA, indicating that at least some SnRK2s are required for stress induced ABA synthesis (Fujii et al., 2011).

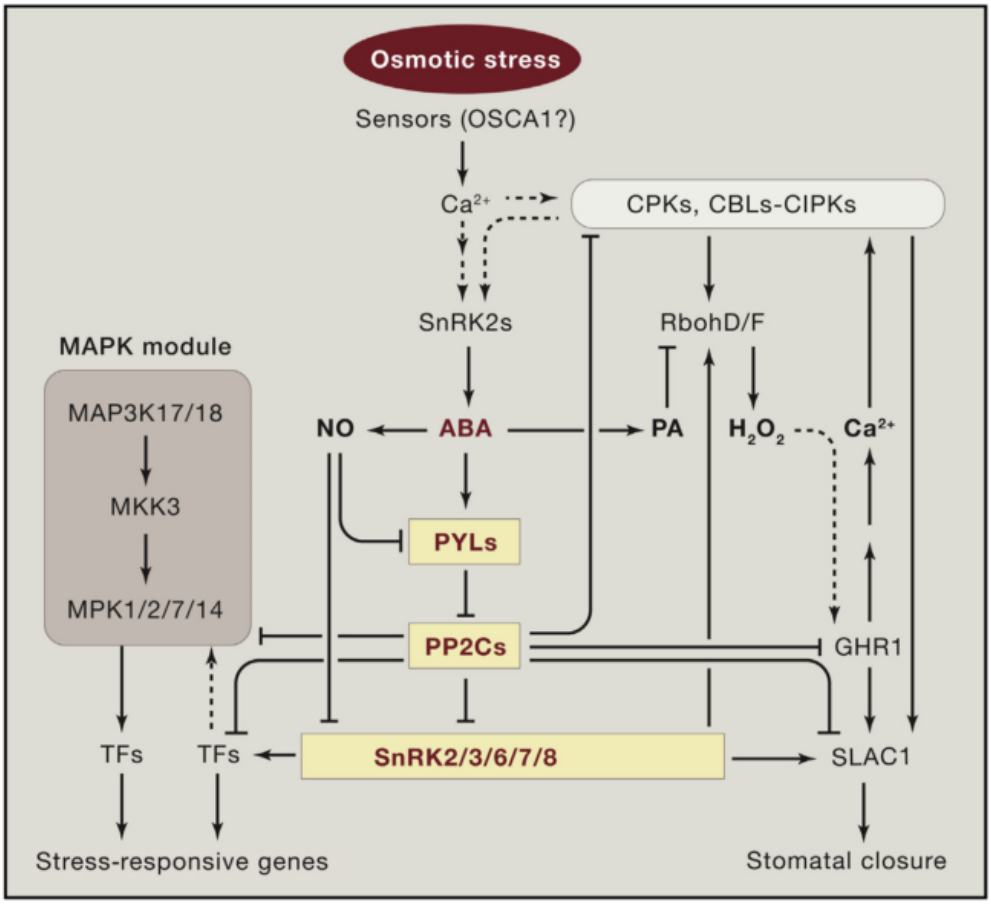

Figure 4: Osmotic stress and ABA sensing and signalling. Although ABA holds a central place in osmotic stress signalling ABA independent pathways exist as well. Figure taken from (Zhu, 2016)

The response to stress also entails major changes of the transcriptome, and transcription factors mediating these stress-induced changes have been identified in several different families (reviewed by Wang et al., 2016). An interesting case is Arabidopsis thaliana activating factor 1 (ATAF1), a NAC transcription factor, which induces the transcription of ABA biosynthetic genes (Jensen et al., 2013). ATAF1 may function at the start of the ABA-dependent pathway, relaying the signal form the ABA-independent pathway.

Mechanisms of stress resistance

Plant responses to stress have been classified into escape, avoidance and tolerance (Levitt, 1980; Kooyers, 2015). Being sessile organisms land plants can of course not physically escape adverse conditions. Rather they “escape” into a stage of their life

cycle which is not susceptible to the stress. For most plants this means reaching reproductive stage and setting seeds, which are metabolically dormant and highly resistant to almost all abiotic stresses. Thus, drought escape responses lead to an acceleration of growth and earlier flowering (Fig. 5).

Avoidance refers to responses which prevent the stress from manifesting within plant cells and consequently avoid deleterious effects on cellular processes. An example of drought avoidance is succulence whereby a plant stores large amount of water. This allows the plant to maintain a high internal water potential regardless of the soil water potential.

Stress tolerance allows the plant to tolerate the effects of the stress, and maintain metabolic functions or at least prevent permanent damage. The most extreme case of drought tolerance is found in resurrection plants which tolerate complete desiccation and can restore normal metabolic functions upon rehydration (Farrant, 2000).

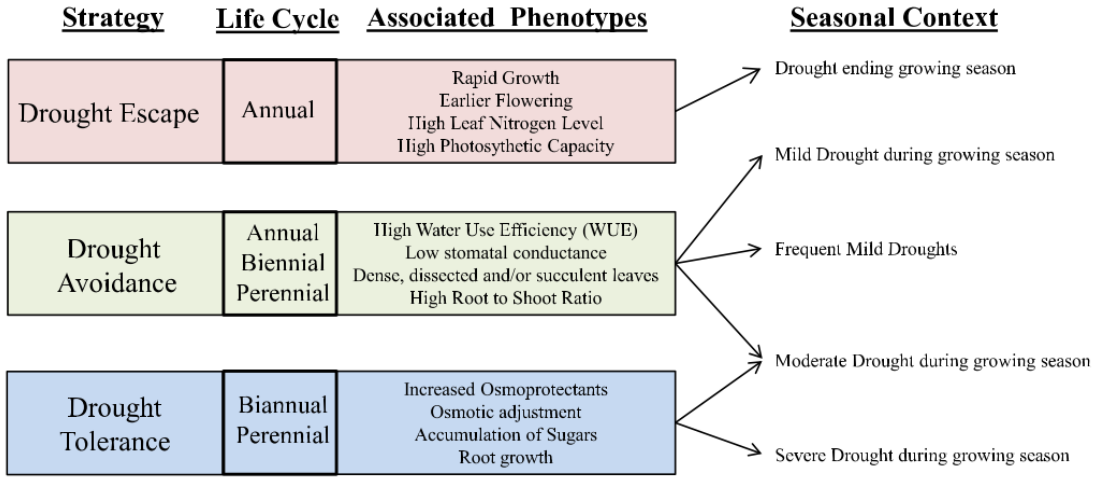

Figure 5: Schematic diagram describing the three mechanisms of drought resistance in herbaceous plants. Figure taken from (Kooyers, 2015)

Although drought escape does increase the fitness of a species in the wild, it is less desirable in agriculture as it leads to lower biomass production and a reduced seed set (Blum, 1996). In contrast, drought avoidance and tolerance are economically valuable traits and there are ongoing attempts to introduce such traits in staple crops through both traditional breeding or genetic engineering (Hu and Xiong, 2014; Luo, 2010). Consequently most research has been devoted to the understanding of these two processes.

Drought avoidance is a key factor of plant productivity during short or moderate drought conditions, and has been investigated intensively (reviewed by Des Marais and Juenger, 2010). Drought avoidance responses often seek to reduce the water loss, allowing the plant to maintain a high water potential. An important short-term

response is the closure of stomata, which preserves water at the cost of limiting CO_2 availability. Upon prolonged exposure to drought, plants will also seek to limit water loss through increasing the thickness of the cuticle (Kosma et al., 2009) and that of the leaf (Karaba et al., 2007). These modification increase the water use efficiency (WUE), i.e. they reduce the amount of water lost for the assimilation of CO_2. In addition to these water-saving changes, plants can also avoid drought by increased water uptake. Drought promotes root growth, which results in an increased root-to-shoot ratio (Chaves et al., 2002; Kooyers, 2015). Although avoidance will allow the plant to maintain a high internal water potential during short periods of drought, it is generally not sufficient to protect against a prolonged severe drought.

Drought tolerance allows plants to cope with reduced internal water potential, such that they can endure long-term drought stress (Verslues et al., 2014). Identification of drought tolerance mechanisms can be confounded by drought avoidance. Drought resistance is often measured by withholding water for a defined period of time. Resistance to such short-term stresses is often conferred by drought avoidance mechanisms, which reduce the water consumption. Thus, plants that show enhanced survival during short-term stresses are not necessarily drought tolerant. For example, analysis of 25 Arabidopsis lines which had been identified as “drought tolerant” revealed that none of them showed an improved growth under prolonged drought (Skirycz et al., 2011). The authors concluded that the previously reported differences in survival rates was due to drought avoidance responses which did not confer tolerance to long-term stress. Therefore, to unambiguously confirm that a mechanism confers drought tolerance it is important to not only analyse responses to short-term stress as in I did in my experiments of chapter 1, but also investigate responses to long-term stress, as was done in chapter 2.

Despite this difficulty, a number of key drought tolerance responses have been identified (reviewed by Verslues et al., 2014).

An important tolerance mechanism is the accumulation of so called “compatible solutes”. These compounds lower the internal osmotic potential of the plant, without disrupting physiological functions. Important compatible solutes are proline, betaines of various amino acids, sugars, and polyols (Slama et al., 2015). Osmotic potential at full turgor is strongly correlated with water availability of natural habitat. Plants adapted to dry environments have a low osmotic potential at full turgor, while plants adapted to well-watered soils – including most crop plants – have a high osmotic potential, highlighting the importance of osmotic adjustment for drought tolerance (Bartlett et al., 2012). The synthesis of these compatible solutes requires alterations of the plant metabolism to provide reduced carbon. As described in chapter 5, starch can be used

as source of carbohydrates during stress, and starch metabolism is affected by many different abiotic stresses.

Another important mechanism conferring drought tolerance is avoidance of damage by reactive oxygen species (ROS) (Chan et al., 2016). Even moderate drought leads to stomatal closure, reducing the amount of CO_2 available for photosynthesis. Without an electron acceptor the electron transport chain becomes over reduced and prone to ROS production (Dietz and Pfannschmidt, 2011; Das and Roychoudhury, 2014). To prevent damages, plants use alternative electron sinks to direct excess reducing potential away from ROS production, but also an intricate system is activated to scavenge and detoxify ROS that have been produced. Excess energy can be dissipated by non-photochemical quenching (NPQ) before reaching the electron transport chain, and mutants deficient in NPQ are more sensitive to abiotic stress (Verhoeven et al., 2001; Havaux and García-Plazaola, 2014). Furthermore, plants use other electron sinks besides the Calvin cycle to relieve the strain on the electron transport chain (Verslues et al., 2014). Interestingly, the synthesis of some compatible solutes like proline and polyols requires reducing power. Mutants deficient in proline synthesis were more sensitive to osmotic stress in general, which can be explained by the absence of the compatible solute, but also suffered from redox imbalance due to the depletion of NADP, which could no longer be regenerated by proline synthesis (Sharma et al., 2011). Thus, both the compatible solute itself and its synthesis protect the plant against drought-induced damages. Mitochondria represent another sink for reducing power and has been suggested to act as a safety valve to dissipate excess energy (Skirycz et al., 2010). If these mechanisms are insufficient to prevent ROS formation, plants can detoxify ROS either enzymatically, e.g. by catalase and superoxide dismutase, or non-enzymatically using antioxidants like ascorbic acid (vitamin C), tocopherol (vitamin E) or glutathione, and mutants lacking either enzymes or antioxidants are more susceptible to a variety of abiotic stresses (reviewed by Das and Roychoudhury, 2014).

Abscisic acid

Structure and functions of ABA

Abscisic Acid (ABA) (Fig. 6) was discovered in the 1960s by several groups independently, both as a fruit abscission promoting substance, explaining the name (Ohkuma et al., 1963) and as a bud dormancy inducing factor (Thomas et al., 1965). Ironically, the function of ABA in abscission and bud dormancy is still debated (Schwartz and Zeevaart, 2004; Dörffling, 2015). Instead, later work revealed that ABA does play a key role in seed development (Nakashima and Yamaguchi-Shinozaki, 2013) and sugar signalling (Rook et al., 2006). In vegetative tissues, ABA is essential for plant survival under abiotic stress, integrating different stress signals and controlling downstream responses. For this reason, ABA is often referred to as a "stress hormone" (Heribert and Shinozaki, 2004). In response to stress ABA levels increase rapidly, as we also show in chapter 1. The elevated ABA content leads to stomatal closure (Kim et al., 2010), changes in gene expression and protein accumulation (Böhmer and Schroeder, 2011), metabolic rearrangements (Kempa et al., 2008), and ultimately changes of plant morphology by increasing root growth while inhibiting shoot growth (Watts et al., 1981).

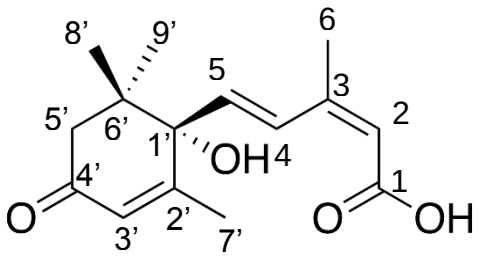

Figure 6: Structure of the naturally occurring isomer of abscisic acid, (+)-S-ABA.

ABA metabolism

In plants ABA is derived from the carotenoid zeaxanthin (Fig 7), the biosynthesis of which was reviewed by (Ruiz-Sola and Rodríguez-Concepción, 2012). Generally, the conversion of zeaxanthin to violaxanthin by ABA1, a zeaxanthin epoxidase (ZEP), is viewed as the first step of ABA biosynthesis (Marin et al., 1996), although this reaction is also involved in other processes, such as non-photochemical quenching. Subsequently, part of the violaxanthin is converted into neoxanthin by ABA4 (North et al., 2007). Both violaxanthin and neoxanthin are in an all-trans form and must be isomerised into their 9-*cis*-isomers for ABA synthesis. However, so far no enzymes catalysing this isomerisation have been identified in Arabidopsis. The first committed step of ABA biosynthesis is the oxidative cleavage of 9-*cis*-violaxanthin or 9-*cis*-neoxanthin by nine-cis-epoxy-carotenoid dioxygenase (NCED), releasing xanthoxin.

Recombinant NCED accepts both 9-*cis*-violaxanthin and 9-*cis*-neoxanthin as substrates *in vitro* (Schwartz et al., 1997b). That said, the reduced ABA content of *aba4* mutants indicates that 9-*cis*-neoxanthin is the preferred substrate *in vivo*. In Arabidopsis nine genes encoding NCEDs have been identified. Different isoforms appear to be expressed in different tissues and under different circumstances. For example NCED5 and NCED6 are highly expressed in the embryo and endosperm during seed development (Tan et al., 2003). During water stress, NCED3 is the most important isoform mediating the accumulation of ABA (Iuchi et al., 2001), and the *nced3* mutant was used as an ABA-deficient mutant in chapter 1.

Xanthoxin is subsequently exported from the plastid to the cytosol where it is step-wise converted into ABA. The first step is the oxidation of the 4'-hydroxyl group to a ketone by ABA2, a short chain dehydrogenase/reductase (SDR). The subsequent opening of the epoxide ring and formation of the 2'-3' double bond is thought to be a spontaneous intramolecular rearrangement. Indeed ABA2 alone is able to convert xanthoxin to abscisic aldehyde *in vitro* (Gonzalez-Guzman, 2002). *aba2* was used as an additional ABA-deficient mutant in chapter 1.

The final step of ABA synthesis is the oxidation of the abscisic aldehyde, which is catalysed by abscisic aldehyde oxidase (AAO). The genome of Arabidopsis encodes four such enzymes (AAO1 to 4). Of these, AAO3 is the isoform required for ABA accumulation in vegetative tissues. Furthermore, AAO3 is also the major isoform in seeds, although AAO1 and AAO4 contribute to ABA synthesis in seeds only (Seo et al., 2004). A peculiar feature of AAOs is their reliance on a sulfurylated molybdenum cofactor. Consequently, mutants lacking the corresponding sulfurase (ABA3) also lack any AAO activity (Schwartz et al., 1997a). However, as the sulfurylated molybdenum cofactor is also used by xanthine dehydrogenase, the *aba3* mutant may show phenotypes unrelated to its ABA deficiency. Therefore *aao3* rather than *aba3* was used as a third ABA-deficient mutant in chapter 1.

ABA synthesis is regulated by controlling the activity of NCEDs, and expression of NCEDs is strongly induced by water stress. In contrast, other biosynthetic enzymes such as ABA2 and AAO3 were detected even in unstressed plants (Endo et al., 2008). However, control of ABA levels requires not only regulation of the synthesis but also inactivation of existing ABA molecules (Finkelstein, 2013).

ABA can be temporarily inactivated by conjugation to other molecules, most commonly glucose (Dong et al., 2014). The resulting glucosyl ester is stored in the vacuole and the apoplast. Upon exposure to water stress, the ester is hydrolysed by β-glucosidase 2 (BG2) directly in the vacuole, or translocated to the endoplasmic reticulum (ER) where hydrolysis is mediated by BG1. The enhanced drought sensitivity of the

corresponding mutants indicates that this remobilisation of ABA plays an important role during stress responses (Xu et al., 2012).

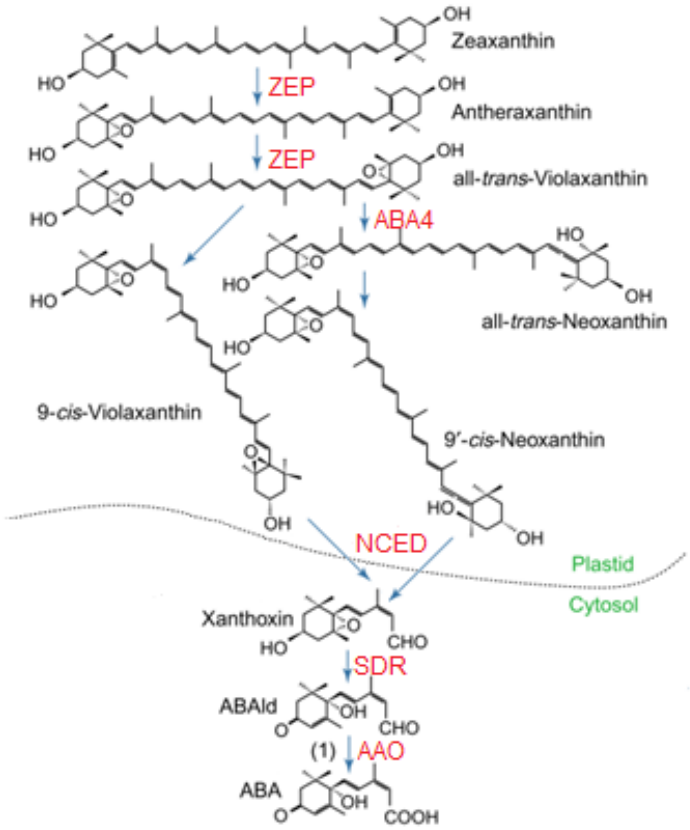

Figure 7: Biosynthesis of ABA from Zeaxanthin. Adapted from (Seo and Koshiba, 2002)

Furthermore, ABA can be degraded through hydroxylation by cytochromes (CYP707A) at the 8' or 9' position, yielding phaseic acid and neo-phaseic acid, respectively (Kushiro et al., 2004; Okamoto et al., 2011). Originally thought to be an inactive catabolite, phaseic acid was recently shown to retain the ability to activate the ABA signalling pathway (Weng et al., 2016). Complete inactivation requires the reduction of the 4' ketone to a hydroxyl group, yielding dihydro-phaseic acid. This reduction is mediated by a dihydroflavonol 4-reductase (DFR)-like enzyme, named phaseic acid reductase (PAR). Subsequently, dihdrophaseic acid is conjugated to glucose and the resulting glucoside is thought to be the end product of ABA metabolism (Weng et al., 2016).

ABA signalling pathways

The elucidation of the ABA signalling cascade has been fraught with difficulties, which in retrospect can be explained by the high redundancy of the pathway. This redundancy masks the effects of loss-of-function mutations, and makes it difficult to investigate the pathway using forward genetics. However, a combination of

pharmacological studies and recombinant systems were successfully used to overcome this redundancy, and the function of the identified genes was corroborated using reverse genetics (reviewed by Finkelstein, 2013).

The core ABA signalling pathway consist of four classes of proteins (Fig 8): pyrabactin resistance 1 (PYR1) and its homologs PYR-like (PYL), which are also known as regulatory components of ABA receptor (RCARs), phosphatases belonging to the PP2C family, SnRKs, and several basic leucine zipper (bZIP) transcription factors (Nakashima and Yamaguchi-Shinozaki, 2013; Fujita et al., 2013). In the absence of ABA, RCARs either exist as monomers (PYL4-10) or form homodimers (PYR1 and PYL1-3). The PP2C phosphatases are active and dephosphorylate SnRKs, leading to the inactivation of SnRKs. Without SnRKs activity, the bZIP transcription factors are likewise dephosphorylated and inactive. Upon addition of ABA, RCARs form a tripartite complex with ABA and PP2Cs, inactivating the phosphatases (Fig. 8). Without the inhibitory effect of PPC2s, SnRKs are activated by phosphorylation of the regulatory loop. The subsequent phosphorylation and activation of bZIP transcription factors by SnRKs leads to the induction of ABA responsive genes. In addition to transcription factors, SnRKs also phosphorylate many other proteins, such as ion channels, modulating their activity (Wang et al., 2013).

RCARs belong to the steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) family (Park et al., 2009). While most members of the START family are involved in lipid or steroid transfer (Alpy and Tomasetto, 2005), the RCAR subfamily evolved into ABA binding receptors (Park et al., 2009; Ma et al., 2009). In Arabidopsis the RCAR family contains 14 homologs, and all but one are able to activate ABA-responsive gene expression in protoplast transfection assays (Fujii et al., 2009). Due to this redundancy, RCAR single mutants show no phenotype, however higher order mutants exhibit gradually increasing ABA insensitivity (Gonzalez-Guzman et al., 2012).

Nonetheless, their different expression patterns indicate that functional differences between the different RCARs exist. Furthermore, it was recently shown that while almost all members of the family can bind ABA, substantial differences exist in their affinity to phaseic acid (Weng et al., 2016).

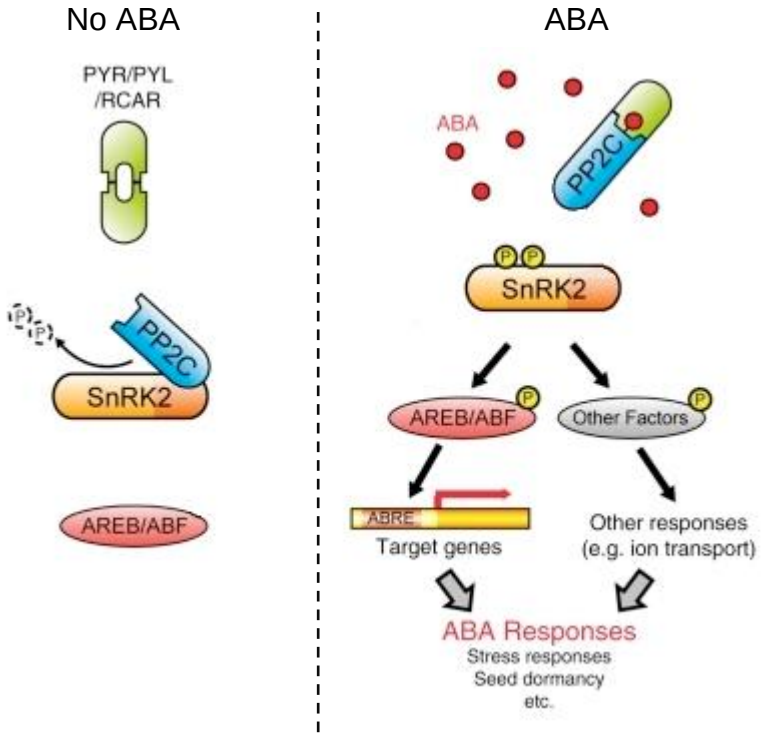

Figure 8: Core signalling pathway of ABA. Adapted from (Umezawa et al., 2010)

PP2C phosphatases act as negative regulators of ABA signalling by dephosphorylating SnRK2s. Therefore, it is surprising that the first two members of the family were identified in a screen for ABA insensitive (ABI) mutants. The ABA insensitive phenotype of *abi1-1* and *abi2-1* is due to a point mutation disrupting the interaction with RCARs. Thus, the activity of the mutated phosphatase can no longer be controlled by ABA, leading to a constitutive dephosphorylation of SnRKs. In contrast, loss of function mutants show the expected ABA hypersensitive phenotype (Saez et al., 2006; Yoshida et al., 2006). ABI1 and ABI2 belong to subfamily A of PP2C phosphatases, which in Arabidopsis consists of 9 members (Komatsu et al., 2009). In addition to ABI1 and ABI2 four other phosphatases have been shown to be involved in ABA signalling (Umezawa et al., 2010).

SnRK2s are important regulators of osmotic stress responses in general, however three kinases – SNRK2.2, SNRK2.3 and SNRK2.6 – are also key components of ABA signalling. Upon being released from inhibition by PP2C phosphatases, SNRKs can either activate themselves by autophosphorylation (Belin et al., 2006) or be activated

through phosphorylation by other kinases (Boudsocq et al., 2007). SNRK2.6 is essential for ABA mediated stomatal closure. After being activated, SNRK2.6 phosphorylates several ion-channels, inhibiting the import of cations and promoting the export of anions (Wang et al., 2013). The resulting efflux of solutes, and consequently water, lowers turgor pressure, leading to stomatal closure. Furthermore, SNRK2.6 also activates NADPH oxidases – respiratory burst oxidase homologs (RBOHs) – which produces ROS as a second messenger involved in stomatal closure (Sierla et al., 2016).

In contrast SNRK2.2 and SNRK2.3 mediate ABA responses in other tissues and during seed germination (Fujii et al., 2007). SNRK2.3 appears to be the most important isoform regulating seed dormancy, while SNRK2.2 seems to be more important during vegetative growth. However, in either case the double mutant displays a more severe phenotype, indicating significant functional overlap between the two isoforms.

Despite the differences between SnRK2.6 on the one hand and SnRK2.2/2.3 on the other hand, some functional redundancy exists as exemplified by the extreme phenotype of the *snrk2.2/snrk2.3/snrk2.6* triple mutant. The triple mutant is completely insensitive to ABA at all stages of development and in all tissues. Furthermore, it is extremely sensitive to water stress and rapidly wilts if exposed to ambient air (Fujii and Zhu, 2009). The triple mutant was used in chapter 1 as an ABA insensitive mutant.

The ABF/AREB/ABI5 family of bZIP transcription factors are activated by SnRK2s-mediated phosphorylation, and are responsible for a majority of ABA-induced changes of gene expression (Kim, 2006). Phosphorylated transcription factors recognize and bind to ABA responsive elements (ABREs) with a consensus sequence of ACGTGGC (Yamaguchi-Shinozaki and Shinozaki, 2005). In order to be induced efficiently by ABA the promoter of a gene needs to contain either two ABREs or an ABRE and a coupling element 3 (CE3) (Hobo et al., 1999). ABI5 has been shown to be a key regulator of ABA responses during seed and seedling development (Lopez-Molina and Chua, 2000; Finkelstein and Lynch, 2000), while at least four homologs mediate ABA-induced changes of transcription in vegetative tissues. As in other steps of the ABA signalling cascades, single mutants show only mild phenotypes, while higher order mutants show more pronounced ABA insensitivity and increased drought sensitivity (Yoshida et al., 2010, 2014a). However, in contrast to the *snrk2.2/snrk2.3/snrk2.6* triple mutants, stomatal responses of *areb1/areb2/abf3* are not impaired, allowing the plant to grow at normal relative humidity (70-80%). The *areb1/areb2/abf3* triple mutant was used as a mutant impaired in ABA-mediated gene expression in chapter 1.

Starch metabolism

Starch is a major carbon storage compound in plants

Starch is an insoluble, chemically inert and osmotically inactive polymer, making it well suited as a storage compound for carbohydrates. Starch is found in all archaeplastida, a group of eukaryotes defined by the presence of a unique organelle derived from a cyanobacterial endosymbiont, the plastid. Interestingly, while starch is found in the cytosol of glaucophytes and rhodophytes (red algae), starch has been relocated to the plastid in chlorophytes (green algae and land plants) (Zeeman et al., 2010; Deschamps et al., 2008). Starch is composed of two molecules, amylose and amylopectin, which together form insoluble starch granules. Both molecules consist of α-glucose units which are linked via α-1,4-glycosidic bonds forming chains, and α-1,6-glycosidic bonds forming branch points. Amylopectin is a highly branched molecule, containing a branch point every 20-25 glucosyl units, while amylose is mostly unbranched. The branches of amylopectin wrap around each other, forming helical structures. This helices cluster together giving rise to the crystalline layers or lamellae of the starch granules, while the branch points of amylopectin form amorphous lamellae (Pfister and Zeeman, 2016). In the starch granule, the amorphous and crystalline lamellae alternate with a periodicity of approximately 9 nm (Zeeman et al., 2002). Amylose is mainly found within the amorphous lamellae and forms single helical structures (Streb and Zeeman, 2012). The amylose content varies greatly between different species and even between different landraces of the same species (Glaring et al., 2006).

In leaves of land plants, the so called “primary or transitory starch” is synthesised during the day and degraded during the night, sustaining the metabolism when photosynthesis is not possible (Zeeman et al., 2007). In contrast, “secondary or storage starch” is used for long-term storage in specialised tissues such as the cereal endosperm or tubers of potato and cassava. This secondary starch is used by humans not only as a food source but also for many industrial purposes (Santelia and Zeeman, 2011).

Synthesis of transitory starch

In the chloroplasts of Arabidopsis leaves, the carbohydrates used for starch synthesis are directly derived from photosynthesis (Fig 9). During the day CO_2 is assimilated through the Calvin cycle using ATP and NADPH produced by the light reaction at the thylakoid membrane. An intermediate of the Calvin cycle, fructose-6-phosphate (Fru6P), is the first metabolite of the starch biosynthesis pathway. Fru6P is isomerised into glucose-6-phosphate (Glc6P) by a plastidial phosphoglucoisomerase (PGI), and

mutants lacking this enzyme have a reduced starch content (Yu, 2000). Glc6P is then converted to glucose-1-phosphate (Glc1P) by a plastidial phosphoglucomutase (PGM). Mutants lacking PGM are completely starch-free in all tissues (Caspar et al., 1985). Glc1P and ATP are the substrates of ADP-glucose pyrophosphorylase (AGPase), which catalyses the formation of ADP-glucose (ADPGlc) and pyrophosphate (PP_i). In contrast the first two reactions which are fully reversible, the formation of ADPGlc is irreversible due to the rapid hydrolysis of PP_i (George et al., 2010) and represents the first committed step of starch synthesis. Consequently, AGPase is tightly regulated to coordinate starch synthesis with the availability of photoassimilates. The AGPase is a heterotetramer consisting of two AGPase small subunits (APS) and two AGPase large subunits (APL). The small subunit is subject to redox regulation and inhibited by the formation of a disulphide bridge (Hendriks et al., 2003; Hädrich et al., 2012). The large subunit is binding two allosteric effectors, 3-phosphoglycerate (3-PGA) and inorganic phosphate, as indicators of CO_2 fixation in the Calvin cycle and energy status, respectively (Crevillén et al., 2003). Furthermore, different APL isoforms (APL1 to 4) exist and the composition of the tetrameric complex affects its kinetic properties, adding another layer of regulation (Crevillén et al., 2005; Ventriglia et al., 2008).

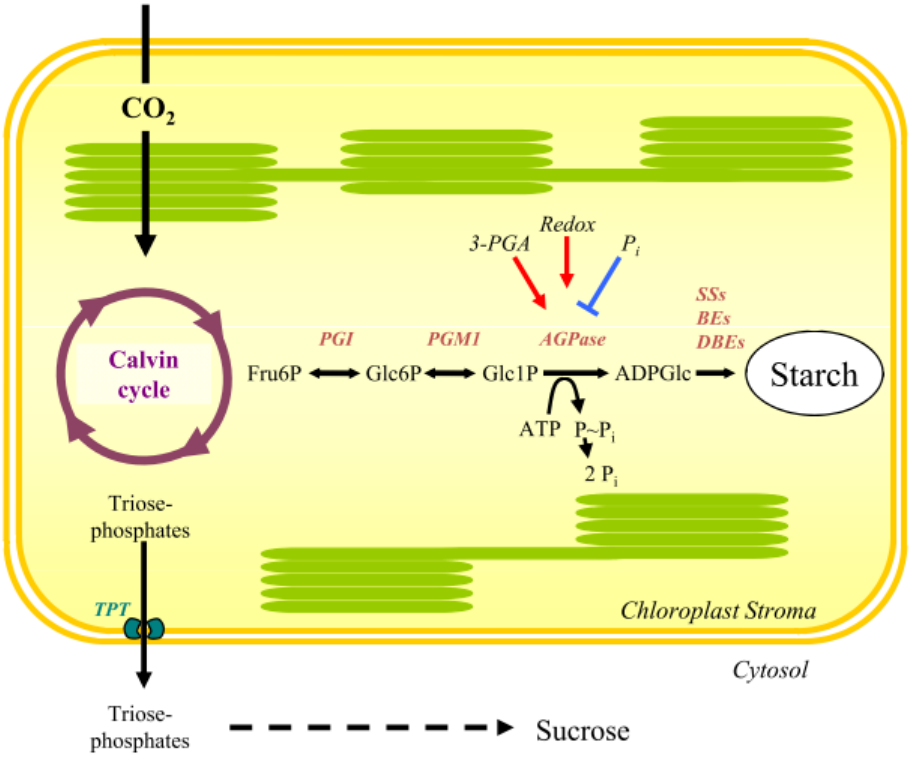

Figure 9: Carbohydrate metabolism during the day in chloroplast of *Arabidopsis thaliana*. Figure taken from (Streb and Zeeman, 2012)

ADPGlc is used as a glucosyl donor by starch synthases (SS1 to 4) and granule bound starch synthase (GBSS), which transfer the glucose to the non-reducing end of an existing α-1,4-linked glucan chain, forming a new α-1,4-glucosidic bond. Although all starch synthases catalyse the same reaction, they differ in their preference to elongate glucan chains of certain length, with SSI preferring short chains, SSII intermediate chains, SSIII long chains, while SS4 is involved in starch granule initiation (Delvallé et al., 2005; Roldán et al., 2007; Zhang et al., 2008; Pfister et al., 2014). While the four starch synthases contribute to the synthesis of amylopectin, GBSS is responsible for the synthesis of amylose, and starch of *gbss* mutants is amylose free (Seung et al., 2015). Mutants lacking a single starch synthase show minor changes in their starch composition, while multiple mutants show more severe phenotypes (Szydlowski et al., 2009). Starch branching enzymes (BE) introduce α-1,6 linked branches by transferring approximately six glucosyl unit-long fragment from the reducing end of a chain to the C6 of a glucosyl unit of another chain. Mutants lacking both branching enzymes cannot synthesise starch and instead accumulate high concentrations of maltose (Dumez et al., 2006). Interestingly, only a minority of the products of BEs can crystallise and form amylopectin, while the reminder forms soluble phytoglycogen instead. The action of a heteromulitmeric isoamylase (ISA) complex is required to convert phytoglycogen into insoluble amylopectin. The complex consist of two proteins, ISA1 and ISA2, both of which are necessary for starch synthesis, and plants lacking either isoform accumulate phytoglycogen instead (Delatte et al., 2005; Pfister et al., 2016). As isoamylases are debranching enzymes (DBE) it is thought that the ISA1ISA2 complex removes branches which impede the crystallisation of amylopectin.

Nocturnal starch degradation

During the night, transitory starch is degraded at a linear rate in a way that ensures that almost all of the starch that was synthesised the previous day is remobilised by the end of the night. The released sugars, mainly maltose and glucose, are exported to the cytosol, where they are further metabolised and used to anabolic reactions during the night (Fig 10). Starch degradation during the night is important for optimal growth. If starch reserves are depleted before dawn, plants enter a phase of starvation, while residual starch at the end of the night leads to non-productive sequestration of carbon. In both cases the resulting perturbations of plant metabolism reduce growth (Stitt and Zeeman, 2012).

In leaves, β-amylases (BAM) are key enzymes involved in starch remobilisation. BAMs are exoamylases which hydrolyse α-1,4-glucosidic linkages at the non-reducing end, releasing the disaccharide maltose. The Arabidopsis genome contains nine genes

encoding β-amylases (BAM1 to 9). BAM3 appears to be the major isoform as the *bam3* mutant shows both a starch excess (*sex*) and reduced maltose levels during the night as well as a substantially lower β-amylases activity (Fulton et al., 2008). BAMs cannot hydrolyse α-1,6-branch points, nor can they release maltose after a branch point. Thus, complete starch degradation requires the presence of debranching enzymes, which hydrolyse branch points, and release linear malto-oligosaccharides. The major isoform of DBEs during nocturnal leaf starch degradation is ISA3. *isa3* mutant shows a starch excess phenotype, and accumulates short glucan chains produced by the action of BAMs at the starch granule surface (Delatte et al., 2006).

The activity of the hydrolytic enzymes is greatly increased by reversible phosphorylation of the starch surface (Edner et al., 2007). This phosphorylation disrupts the double helices of amylopectin glucan chains, thereby solubilizing the granule surface, rendering it more accessible to the hydrolytic enzymes (Blennow and Engelsen, 2010). Starch phosphorylation is mediated by glucan water dikinase (GWD) and phosphoglucan water dikinase (PWD), which phosphorylate glucosyl residues in starch at the C6 and the C3 position, respectively.

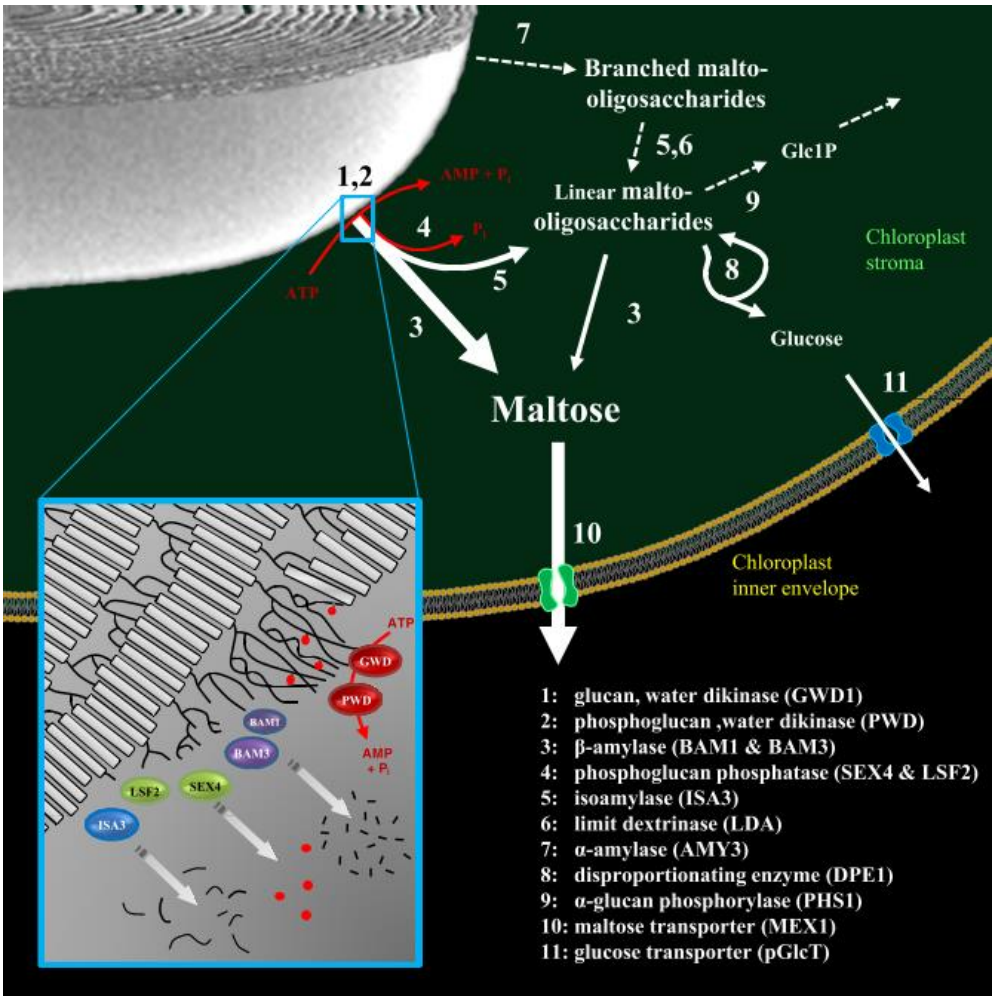

Figure 10: Night-time starch degradation in the chloroplast of *Arabidopsis thaliana*. Figure taken from (Streb and Zeeman, 2012)

Approximately 1 in 200 glucosyl residues in amylopectin is phosphorylated, with C6 phosphorylation being the more abundant modification (Ritte et al., 2006; Santelia et al., 2011). The importance of reversible phosphorylation is demonstrated by the severe starch excess and growth reduction observed in *gwd* mutants (Yu et al., 2001; Ritte et al., 2002), as well as the milder starch excess observed in the *pwd* mutant (Kötting et al., 2005).

As BAMs cannot degrade past a phosphorylated glucosyl residue, complete starch degradation also requires the activity of phosphoglucan phosphatases. The most important phosphoglucan phosphatase is SEX4, which catalyses the removal of both C6 and C3 linked phosphate groups, although with an apparent preference for the C6 position (appropriate ref). In *sex4* mutants starch degradation is impaired resulting in a starch excess and the accumulation of soluble phosphorylated glucans (Zeeman et al., 1998; Kötting et al., 2009).

Besides maltose, the combined action of BAMs and debranching enzymes also leads to the formation of maltotriose which cannot be degraded further by BAMs. The action of an additional enzyme is required here: the disproportioning enzyme 1 (DPE1), DPE1 is a α-1, 4 glucanotransferase, which transfers a two glucose units from maltotriose to another soluble oligosaccharide, while releasing glucose. *In vivo* the primary acceptor of the transferred maltose is another maltotriose molecule. The accumulation of maltotriose in the *dpe1* mutant results in a starch excess phenotype, most likely through a feedback inhibition on starch degradation (Critchley et al., 2001; Li et al., 2017).

Apart from the abovementioned enzymes, catalytically inactive proteins such as BAM4 (Fulton et al., 2008) and LSF1 (Comparot-Moss et al., 2010) are also involved in starch degradation, although their exact function is so far unknown. It is possible that they are regulatory proteins or facilitate the binding of active enzymes to starch (Li et al., 2009).

Regulation of diel leaf starch metabolism

Although starch synthesis and starch degradation are induced by illumination and darkness, respectively, the photoperiod and the time of illumination or darkness as measured by the circadian clock also impact on starch metabolism (Graf et al., 2010; Scialdone et al., 2013; Mugford et al., 2014). Under short photoperiods (e.g. 8 h light, 16 h dark), starch synthesis occurs at a higher rate to ensure accumulation of adequate reserves by the end of the day, while degradation is slowed down to ensure the starch reserves last throughout the night and carbon starvation is avoided (Gibon et al., 2009). This mechanism is resilient to environmental perturbations such as unexpectedly early nightfall (Scialdone et al., 2013) or changes in night-time

temperature (Pyl et al., 2012). Control over nocturnal starch degradation depends on the circadian clock as demonstrated by the premature exhaustion of starch reserves in the *cca1/lhy* double mutant, which is deficient in two core components of the circadian clock (Graf et al., 2010). However, the precise mechanism has not been elucidated to date. Although the transcript abundance for many genes involved in starch metabolism shows strong oscillations over the diurnal cycle (Smith et al., 2004), this is often not reflected by changes in protein abundance (Lu et al., 2005). Thus, it appears that the control of the rate of starch degradation occurs mostly at the posttranslational level. While it is not known how the information of the circadian clock about the expected night length is transduced to the starch degrading machinery, the target appears to be PWD. The *pwd* mutants were no longer able to adjust starch degradation to changes in day-length (Scialdone et al., 2013). Interestingly, this function is exclusive to PWD as mutants deficient in the other glucan phosphorylating enzyme GWD adjusted their starch degradation rates normally (Skeffington et al., 2014).

Starch synthesis appears to be regulated mainly at the level of the AGPase through both redox and allosteric regulation (Gibon et al., 2004; Mugford et al., 2014), and depends on gigantea (GI) and flavin binding, kelch repeat, F box1 protein (FKF1), two transcription factors associated with photoperiod signalling and the circadian clock (Mugford et al., 2014).

The Arabidopsis genome encodes more enzymes than are required for diel starch turnover

In addition to the abovementioned enzymes which are required for night-time starch degradation, the Arabidopsis genome contains several of their homologs which appear to be dispensable for growth under standard conditions. For example, only two of the nine β-amylases are required for night-time starch degradation (Fulton et al., 2008). Furthermore, several gene families, which have been shown to be involved in starch degradation in other species, are also found in Arabidopsis, but they appear not to participate in night-time starch degradation. One such enzyme is α-amylase (AMY). AMYs are endoamylases and hydrolyse α-1,4-linkages releasing linear and branched oligosaccharides. AMYs are key enzymes of storage starch remobilisation in the cereal endosperm (Fincher, 1989), and silencing of AMY genes by RNAi causes strong growth retardation in rice seedlings (Asatsuma et al., 2005). The genome of Arabidopsis encodes for three AMYs. However, since members of the Brassicaceae family, including Arabidopsis, use primarily fatty acids as a carbon storage in their seeds (Li et al., 2006), an involvement of α-amylases in seed germination is unlikely.

Analysis of mutants deficient in one or multiple AMYs revealed that the diel starch turnover in Arabidopsis does not rely on AMYs (Yu et al., 2005). α-glucan-phosphorylases (PHS or PHO) degrade starch by a phosphorolytic cleavage of α-1,4-linkages at the reducing end of a glucan chain, releasing Glc1P. Phosphorylases have been implicated in storage starch remobilisation in the cotyledons of legumes (Bewley et al., 2013) or tubers of sweet potatoes (Hagenimana et al., 1994). The genome of Arabidopsis encodes two phosphorylases (PHS1 and PHS2), but both *phs1* and *phs2* mutant plants degrade starch normally during the night (Zeeman et al., 2004; Schopper et al., 2015).

Not all homologs of starch degrading enzymes are necessarily involved in starch metabolism. Indeed, a number of proteins localise to a compartment other than the chloroplast, e.g. GWD2, PHS2, and BAM5 are cytosolic proteins (Pirone et al., 2017; Schopper et al., 2015; Laby et al., 2001), AMY1 is secreted (Doyle et al., 2007), and BAM7 and BAM8 are nuclear proteins (Reinhold et al., 2011). However, other genes belonging to these families have been demonstrated to be active amylases participating in starch degradation *in vivo*. For example, the *bam3* mutant exhibits only a mild starch excess phenotype, which is much more pronounced in the *bam1/bam3* double mutant, although the *bam1* single mutant degrades starch normally (Fulton et al., 2008). This indicates that the two BAMs have partially overlapping roles and that BAM1 is an active amylase *in vivo*. Similarly, although the *amy3* single mutant degrades starch normally, the loss of AMY3 does aggravate the starch excess of *isa3* and *isa3/lda* mutants (Streb et al., 2012) as well as of *sex4* mutants (Kötting et al., 2009). Therefore, AMY3 can contribute to transitory starch degradation in plastids. Interestingly, it appears that AMY3 is also active during the day. The loss of all debranching enzymes prevents starch accumulation in favour of phytoglycogen as demonstrated by the *isa1/isa2/isa3/lda* mutant. However, the subsequent loss of AMY3 restores starch synthesis (Streb et al., 2008). Similar results were obtained analysing mutants lacking SS3 and SS4. Starch synthesis is reduced in *ss4* mutants and almost absent in *ss3/ss4* double mutants, but restored in *ss4/amy3* and *ss3/ss4/amy3* mutants (Seung et al., 2016).

Although these studies demonstrate that BAM1 and AMY3 are active enzymes capable of degrading starch *in vivo*, they did not elucidate the function of these enzymes in wild-type plants.

Light-induced starch degradation in guard cells

It has been known for a long time that starch metabolism in guard cells follows a different pattern than in mesophyll cells (Lloyd, 1908). While starch reserves are

depleted at the end of the night in mesophyll cells, guard cells retain high amount of starch at dawn. After illumination starch in guard cells is rapidly degraded, while in mesophyll cells starch is synthesised. Stomatal opening was impaired in the starchless *pgm* mutant, indicating that the observed starch degradation in wild type plants is important for stomatal function (Lasceve et al., 1997). More recently, the development of new method for starch quantification in single cells allowed a detailed analysis of starch metabolism in guard cells (Horrer et al., 2016; Horrer, 2016). It was shown that starch degradation in guard cells relies on a different set of enzymes than nocturnal starch degradation. In particular BAM1 and AMY3 were identified as key enzymes, and the *amy3/bam1* double mutant was impaired not only in starch degradation but also stomatal opening (Horrer et al., 2016).

Therefore, it appears that some starch degrading enzymes which do not participate in nocturnal starch degradation, instead degrade starch in other tissues and during other conditions.

Role of starch degradation during abiotic stress

The main objective of my PhD was to investigate the role of starch during abiotic stress, and as part of this aim, I conducted an extensive analysis of the existing literature, which is presented in chapter 5. I found that numerous studies reported major changes of starch metabolism during different types of abiotic stress in many different species. Briefly, drought, osmotic and heat stress as well as exogenous ABA induced a reduction of starch content in a majority of studies, while cold and salt stress were often associated with increased starch content (Thalmann and Santelia, 2017). These changes appear to be conserved in the plant kingdom as they are found in green algae, basal land plants like mosses, and flowering plants. Thus, rearrangements of carbohydrate metabolism appears to be an integral part of plant stress response. Furthermore, the stress-induced changes correlated with plant resistance to abiotic stress, and ecotypes or landraces exhibiting greater changes were more resistant to stress (González-Cruz and Pastenes, 2012).

Employing reverse genetics studies in Arabidopsis identified some enzymes involved in stress-induced starch degradation, and also highlighted differences between different types of stress. For example, cold stress induces *BAM3* expression, while heat or drought stress induces the expression of *BAM1*. In line with these results, plants lacking BAM1 or BAM3 accumulated more starch during osmotic or cold stress, respectively (Kaplan and Guy, 2005; Valerio et al., 2011).

AIM OF THIS THESIS

The three major goals of this thesis were: (1) to identify the enzymes involved in osmotic stress-induced starch degradation; (2) to elucidate the regulatory mechanisms controlling stress-induced starch degradation; (3) to investigate the physiological role of stress-induced starch degradation. To address these questions it was necessary to establish a method to reliably induce water stress in a defined manner. After it became clear that osmotic stress in hydroponics allowed for a more controlled induction of stress compared to water stress in soil, further experiments were conducted in hydroponics. Amylases involved in stress-induced starch degradation were identified by transcriptome analysis, and two candidates, BAM1 and AMY3, were selected for further investigation based on previous evidence of their involvement in starch degradation during the light. Their role in stress-induced starch degradation was verified using knock-out mutants. Secondly, the promoters of *BAM1* and *AMY3* were analysed for *cis*-regulatory elements involved in stress-responsive gene expression, revealing the presence of ABA responsive elements in both genes. ABA responsiveness of both genes was verified using several mutant lines and application of exogenous ABA. Finally, the fate of sugars released from starch during osmotic stress was investigated using a labelling technique, revealing an important role of starch degradation in root growth under stress. These results are presented in chapters 1 and 2.

BAM1 was reported to be subject to post-translational regulation, such as phosphorylation and formation of disulphide bridges. Therefore we also tried to investigate if these mechanisms contributed to the regulation of BAM1 activity during osmotic stress. The results are presented in chapter 3.

As night-time starch degradation is influenced by the circadian clock we also investigated the influence of the circadian clock on stress-induced starch degradation. Preliminary results are presented in chapter 4.

Lastly, we also investigated if the mechanisms of stress-induced starch degradation were conserved in other species. To address these questions we reviewed existing literature about starch and carbohydrate metabolism during abiotic stress in different species. The results of this survey are presented in a form of a review in chapter 5. Furthermore, we conducted an extensive phylogenetic analysis of β-amylases to see if BAM1 and key regulatory elements in its promoter were also present in other plants. The results are presented in chapters 1 and 6.

1 – Regulation of leaf starch degradation by abscisic acid is important for osmotic stress tolerance in plants

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Synopsis: In this report, we identify BAM1 and AMY3 as key enzymes of stress-induced starch degradation, and show that their expression is induced by both osmotic stress (300 mM mannitol for 4 hours) and ABA (100 µM for 4 hours). The *amy3bam1* double mutant failed to degrade starch in response to osmotic stress and was more sensitive to stress. Mutants impaired in either ABA synthesis (*aao3*, *aba2*, *nced3*) or ABA signalling (*snrk2.2*/*snrk2.3*/*snrk2.6, areb1/areb2/abf3*) were impaired in stress-induced starch degradation. We could show that the sugars released from starch are converted into sucrose and exported to the roots. This export promotes water uptake and root growth under osmotic stress.

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Regulation of Leaf Starch Degradation by Abscisic Acid Is Important for Osmotic Stress Tolerance in Plants[OPEN]

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Starch serves functions that range over a timescale of minutes to years, according to the cell type from which it is derived. In guard cells, starch is rapidly mobilized by the synergistic action of β-AMYLASE1 (BAM1) and α-AMYLASE3 (AMY3) to promote stomatal opening. In the leaves, starch typically accumulates gradually during the day and is degraded at night by BAM3 to support heterotrophic metabolism. During osmotic stress, starch is degraded in the light by stress-activated BAM1 to release sugar and sugar-derived osmolytes. Here, we report that AMY3 is also involved in stress-induced starch degradation. Recently isolated *Arabidopsis thaliana amy3 bam1* double mutants are hypersensitive to osmotic stress, showing impaired root growth. *amy3 bam1* plants close their stomata under osmotic stress at similar rates as the wild type but fail to mobilize starch in the leaves. ^{14}C labeling showed that *amy3 bam1* plants have reduced carbon export to the root, affecting osmolyte accumulation and root growth during stress. Using genetic approaches, we further demonstrate that abscisic acid controls the activity of BAM1 and AMY3 in leaves under osmotic stress through the AREB/ABF-SnRK2 kinase-signaling pathway. We propose that differential regulation and isoform subfunctionalization define starch-adaptive plasticity, ensuring an optimal carbon supply for continued growth under an ever-changing environment.

INTRODUCTION

Starch is the most abundant form in which plants store carbohydrates. Its metabolism and function depends upon the cell type from which it is derived. In guard cells, starch is present at night and degraded within 30 min of light to promote rapid stomatal opening (Horrer et al., 2016; Blatt, 2016). In the leaves, starch typically accumulates gradually during the day using a fraction of the carbon assimilated through photosynthesis. At night, the starch that was synthesized the previous day is almost precisely consumed at dawn for continued sucrose biosynthesis and energy production when photosynthesis does not occur, a process vital for plant growth (Smith and Stitt, 2007; Stitt and Zeeman, 2012; Scialdone and Howard, 2015; Graf and Smith, 2011). Mutant *Arabidopsis thaliana* plants that fail to synthesize or degrade starch in the leaves have reduced growth rates under most conditions (Yazdanbakhsh and Fisahn, 2011; Usadel et al., 2008b). This nearly linear pattern of starch biosynthesis and degradation is retained under changing photoperiods or if plants are subject to a sudden early or late dusk, as long as the total circadian rhythm remains at 24 h (Sulpice et al., 2014; Graf et al., 2010). It is indeed observed that plants degrade starch faster in long days than in short days, demonstrating that plants somehow anticipate the length on the following night (Gibon et al., 2004; Lu et al., 2005). Such a tight regulation of starch degradation rates prevents carbon starvation or nonproductive carbon sequestration, thereby supporting continued growth during the night (Stitt and Zeeman, 2012).

Evidence is accumulating for an analogous adaptive response of leaf starch metabolism to other challenges, such as a severe water deficit or extreme temperatures. In response to acute temperature shock, plants mobilize starch at time when biosynthesis would be expected (e.g., in the middle of the light period), resulting in the accumulation of maltose, the major starch catabolite, and of its deriving sugars (Usadel et al., 2008a; Purdy et al., 2013; Kaplan and Guy, 2005, 2004; Sitnicka and Orzechowski, 2014; Yano et al., 2005; Kaplan et al., 2007). Similar rearrangements of starch metabolism are observed when plants are subject to short periods of oxidative or osmotic stress (Scarpeci and Valle, 2008; Zanella et al., 2016; Valerio et al., 2011; Geigenberger et al., 1997). It is proposed that soluble sugars and other charged metabolites, such as proline or glycine, may function as osmoprotectants during stress responses. Stress-induced accumulation of these metabolites lowers the water potential of the cell, promoting water retention in the plant without interfering with normal metabolism. This process, known as osmotic adjustment, enables the maintenance of cell turgor for plant growth and survival under stress conditions (Bartels and Sunkar, 2005; Verslues and Sharma, 2011; Krasensky and Jonak, 2012). Sugars and proline can also help stabilize proteins and cell structures, particularly when the stress becomes severe or persists for

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longer periods (Hoekstra et al., 2001). These compounds can also act as free radical scavengers, protecting against oxidation by removing excess reactive oxygen species, reestablishing the cellular redox balance (Couée et al., 2006; Miller et al., 2010). Thus, the ability to adjust patterns of assimilation, storage, and utilization of carbon in response to changes in the environment may determine not only biomass production but also plant fitness in terms of survival under stressful environmental conditions. Despite its importance, our understanding of how carbon is provided for metabolism and growth under stress is poor.

Transitory starch degradation at night begins with the phosphorylation of the glucan chains by glucan, water dikinase (GWD) and phosphoglucan, water dikinase (PWD) (Ritte et al., 2006). The chains are then simultaneously degraded by a set of glucan-hydrolyzing enzymes (including β-amylases [BAM], α-amylases [AMY], and debranching enzymes) and dephosphorylated by phosphoglucan phosphatases (Streb and Zeeman, 2012). These enzymes work in synergy to completely degrade starch (Kötting et al., 2009; Edner et al., 2007). Hydrolysis of starch to maltose by BAM represents the predominant pathway of transitory starch degradation. BAM3 is the major isoform in the nighttime leaf starch metabolism, and Arabidopsis *bam3* mutants have elevated amounts of starch and reduced levels of maltose at night compared with the wild type (Fulton et al., 2008; Kaplan and Guy, 2005). BAM1 is highly expressed in guard cells and, in synergy with AMY3, degrades starch in these cells for light-induced stomatal opening (Horrer et al., 2016; Blatt, 2016). Under osmotic stress, BAM1 is activated in the leaves and contributes to diurnal starch degradation for sugars and proline biosynthesis (Valerio et al., 2011; Zanella et al., 2016). Thus, it appears that BAM1 is important for stress-induced leaf starch degradation. However, it is unclear if other starch hydrolytic enzymes also play a role during stress, how the process is controlled, and what the significance is for plant stress tolerance.

To address these questions, we subjected hydroponically grown Arabidopsis plants to a short-term, high osmotic stress and show that AMY3 is also involved in stress-induced starch degradation. Mutants lacking both BAM1 and AMY3 are sensitive to osmotic stress. This is because in the absence of these two enzymes, plants fail to mobilize starch in the leaves during stress and have reduced carbon export to the root, affecting osmolyte accumulation for water and nutrient uptake and root growth. We also show that the stress hormone abscisic acid (ABA) controls the activity of BAM1 and AMY3 during stress responses, and we provide evidence that the mechanism we describe for Arabidopsis is most likely conserved among different plant species. Our discovery uncovers a critical function for starch in plant stress tolerance and highlights BAM1 and AMY3 as targets for breeding stress-tolerant crops.

RESULTS

BAM1 and AMY3 Synergistically Degrade Starch in Leaves upon Osmotic Stress

To study the effects of osmotic stress on starch metabolism, we subjected 3-week-old Arabidopsis plants grown in hydroponic culture to 300 mM mannitol treatment for 4 h, starting after 3 h of light. The plants visibly wilted but, when returned to control solution without mannitol for 24 h, they recovered fully (Figure 1A). After 4 h of stress, wild-type plants accumulated 51% less starch compared with control plants (Figure 1B). The reduced accumulation of starch was accompanied by a significant accumulation of maltose to levels comparable to, or higher than, those normally observed at night (Figure 1C) (Fulton et al., 2008). The plants responded in a similar way when the osmotic stress was imposed using 300 mM sorbitol (Supplemental Figure 1). The Arabidopsis mutant *phosphoglucomutase* (*pgm*), which is devoid of starch (Caspar et al., 1985), accumulated tiny amounts of maltose during the day under control growth conditions. However, in response to osmotic stress, the maltose levels in *pgm* remained unchanged (Supplemental Figure 2). Thus, the reduction in starch accumulation in osmotically stressed plants appeared, at least in part, to result from starch turnover (i.e., simultaneous biosynthesis and degradation).

BAM1 gene expression was induced 8-fold in wild-type leaves upon mannitol treatment, similar to *RD29A*, a well-known osmotic stress-responsive gene (Figure 1D) (Yamaguchi-Shinozaki and Shinozaki, 1994). *bam1* mutant plants showed reduced osmotic stress-induced starch degradation and maltose accumulation but were indistinguishable from the wild type under control conditions (Figures 1B and 1C; Supplemental Figure 3). In contrast, *BAM3* transcripts were not induced by mannitol (Figure 1D), and *bam3* mutants, despite having elevated starch levels compared with the wild type, activated starch degradation similarly to the wild type (Figures 1B and 1C; Supplemental Figure 3). *BAM1* gene expression was stress-induced in *bam3* mutants to the same extent as in the wild type (14-fold in this experiment; Supplemental Figure 4), suggesting that BAM1 is activated in *bam3* to promote starch mobilization during the osmotic stress response. These results indicate that BAM1 and BAM3 are critical under different conditions.

The finding that BAM1 degrades starch synergistically with AMY3 (Seung et al., 2013; Horrer et al., 2016) prompted us to investigate the relevance of this interaction during osmotic stress responses. Like *bam1*, the *amy3* mutant appears similar to the wild type under standard growth conditions (Supplemental Figure 3) (Yu et al., 2005). However, upon stress, *amy3* mutants showed a reduction in starch degradation and reduced maltose accumulation relative to the wild type, showing that AMY3 is also required for starch degradation under osmotic stress (Figures 1B and 1C). The defects in *amy3* were not as severe as those in *bam1*. qPCR analysis of *AMY3* expression under osmotic stress showed that there was a small induction after 4 h of mannitol treatment (Figure 1D). This induction was in addition to the previously described increase in AMY3 transcript that occurs during the day (apparent in our control plants; Figure 1D; Supplemental Figure 5A) (Smith et al., 2004). Loss of both AMY3 and BAM1 proteins in the *amy3 bam1* double mutant did not alter starch levels under osmotic stress, with starch accumulating to the same extent as in unstressed plants (Figures 1B and 1C). However, starch metabolism under control conditions was not affected in this mutant (Supplemental Figure 3) (Horrer et al., 2016). These results suggest that BAM1 and AMY3 are both induced during osmotic stress and work together to mediate efficient starch catabolism in the leaves.

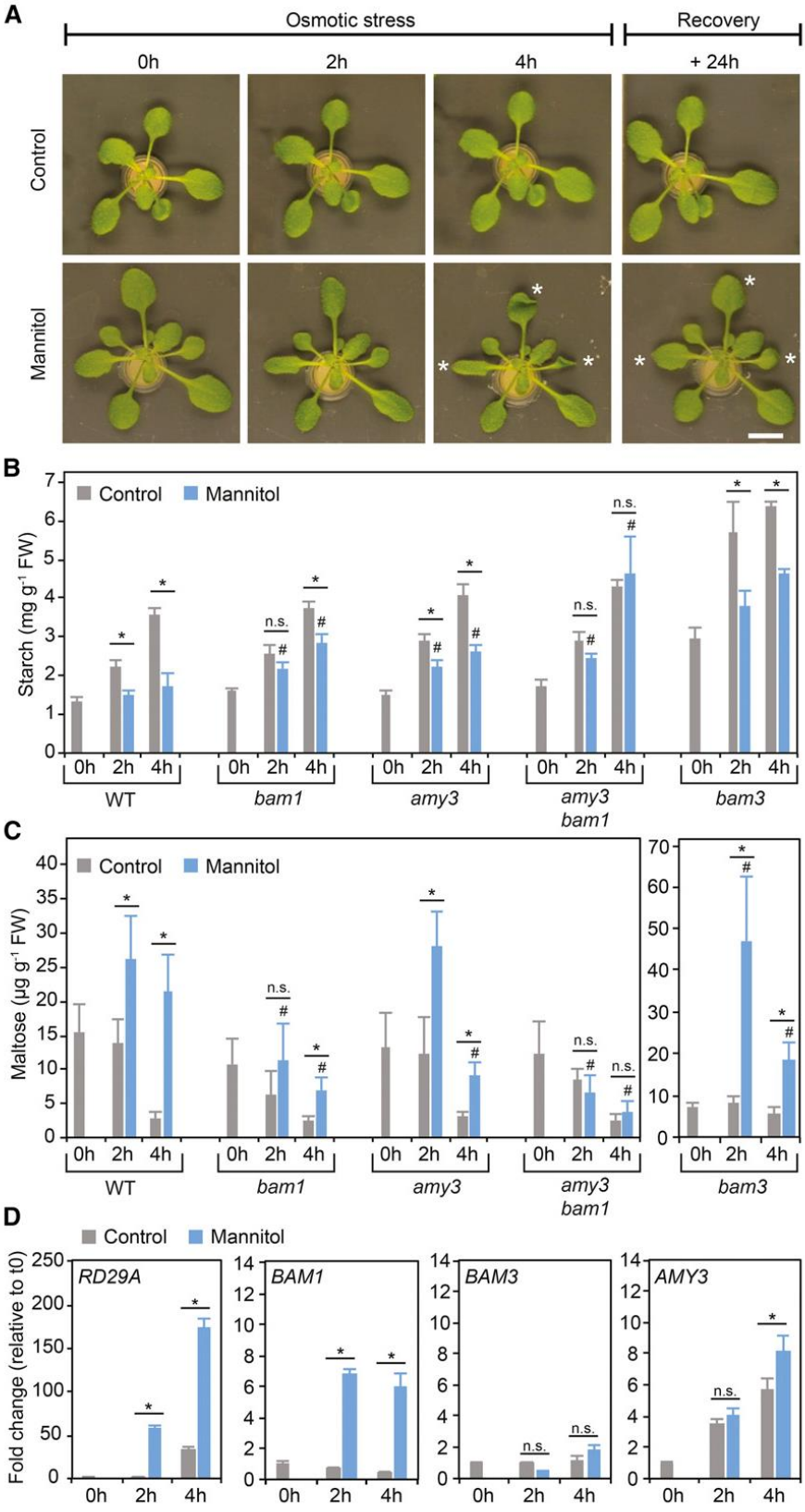

Figure 1. Leaf Starch Levels during Osmotic Stress Are Unchanged in *amy3 bam1* Mutant Plants.

(A) Three-week-old hydroponically grown Arabidopsis plants were transferred to a nutrient solution optionally supplemented with 300 mM mannitol for 4 h. After the stress treatment, roots were rinsed with water and returned to a control solution for 24 h. Representative wild-type plants (Col-0) show the reduction in turgor in response to mannitol stress (denoted by asterisks) from which the plants recovered after 24 h in control nutrient solution. Bar = 1 cm.

Simultaneous Loss of BAM1 and AMY3 Affects Osmotic Stress Tolerance

To investigate the importance of starch degradation in osmotic stress tolerance, we examined *amy3 bam1* performance under stress. Measurements of leaf relative water content revealed that *amy3 bam1* mutant plants lost water more quickly than the wild type. After 1 h of stress, the water content in *amy3 bam1* had already decreased by 25% relative to the control, whereas the wild type only lost 7% of water (Figure 2A). Leaf sap osmolality increased in parallel with the loss of water, and by the end of the stress treatment, *amy3 bam1* showed an increase of 44% compared with a 29% increase in the wild type (Figure 2B). Furthermore, less water was absorbed and transpired by *amy3 bam1* under stress than by the wild type (only 63 and 79% as much after 2 and 3 h of mannitol treatment, respectively; Supplemental Figure 6). We recently reported that stomatal opening is impaired in *amy3 bam1* mutants due to the constitutive high levels of guard cell starch in these plants (Horrer et al., 2016). However, despite the differences in stomatal width (reduced in *amy3 bam1*), both the wild type and *amy3 bam1* closed their stomata at similar rates upon transfer to a mannitol-containing solution (Figure 2C). Thus, the rapid loss of water in *amy3 bam1* during the first hour of mannitol treatment cannot be explained by differences in stomatal closure. If transpiration through stomata were the main determinant of *amy3 bam1* responses to osmotic stress, a reduced rather than an increased loss of water would be expected. It is more likely that the reduced water absorption ability of *amy3 bam1* affected the water content in the leaves.

To assess the possibility that underground plant parts are involved in osmotic stress responses in *amy3 bam1*, we applied the stress to seedlings grown on half-strength Murashige and Skoog (MS) vertical agar plates. Six days (6 d) after germination, seedlings with the same root lengths were transferred for an additional 9 d to agar plates optionally supplemented with 300 mM mannitol. Primary root growth was reduced in the wild type upon stress. However, *amy3 bam1* showed a higher degree of inhibition (Figure 2D). Fresh weight measurements after 9 d of stress treatment showed that *amy3 bam1* and wild-type root growth was inhibited by 48 and 61%, respectively, relative to the controls (Figure 2E). Interestingly, shoot growth inhibition was similar for both genotypes (~63 to 66% of control; Figure 2E), resulting in substantial differences in root to shoot ratios. Wild-type plants increased their root to shoot ratio by 75% relative to the controls, whereas in *amy3 bam1*, the ratios remained unaltered (Figure 2F). Altogether, these results suggest that AMY3/BAM1-mediated leaf starch degradation contributes to osmotic stress tolerance by affecting root growth and function in response to stress.

Carbon Export to the Root and Osmolyte Accumulation during Osmotic Stress Are Reduced in *amy3 bam1*

The root phenotype of *amy3 bam1* suggests that stress-induced starch degradation affects the metabolism of the plant as whole. We therefore used $^{14}CO_2$ labeling to analyze carbon partitioning into different cellular compound classes and to measure carbon export from leaves to the roots in hydroponically grown plants with or without mannitol. We supplied $^{14}CO_2$ to the whole plant for 1 h, either at the beginning of the stress (after 3 h of light) or in the middle of the stress treatment (after 5 h of light). In each case, the $^{14}CO_2$ pulse was followed by 1 h chase in air (Figure 3A), after which the rosettes and roots were harvested separately. Under control conditions, carbon partitioning in wild-type and *amy3 bam1* plants was very similar (Supplemental Tables 1 and 2). Under osmotic stress, however, the two genotypes differed markedly in the amount of carbon channeled to the root. While the wild type maintained a high rate of carbon export during the whole experiment, *amy3 bam1* showed no increase in ^{14}C export after 4 h of stress (Figure 3B). Subfractionation of root-soluble compounds revealed that most of the imported carbon was present in neutral compounds (i.e., sugars), with no significant changes in the basic and acidic fractions (Figure 3C). Consistent with reduced carbon export to the root, the amount of label found in neutral sugars after 4 h of stress in *amy3 bam1* roots was reduced compared with the wild type (Figure 3C). Conversely, carbon allocation within the leaf soluble fraction was similar between the two genotypes, showing an increase in neutral sugars and a reduction in basic and acidic compounds (Figure 3D). This was accompanied by a decrease in carbon partitioning into starch and cell wall (Figure 3E). Interestingly, even though the labeling of starch and cell wall in *amy3 bam1* leaves after 4 h of stress was unchanged compared with the controls (Figure 3E), the amount of carbon allocation into leaf soluble sugars was still elevated relative to the unstressed control plants, as with the wild type (Figure 3E). This suggests that most of these sugars derive from photosynthetic carbon assimilation and only partly from starch hydrolysis. Alternatively, *amy3 bam1* might suffer from reduced sugar phloem loading.

Consistent with the ^{14}C-labeling experiments, we found that wild-type plants under osmotic stress accumulated much higher levels of soluble sugars, mostly sucrose, both in leaves and roots (Figures 4A to 4C; Supplemental Figures 7A to 7C). Proline also accumulated in high amounts toward the end of the stress treatment (Figure 4D; Supplemental Figure 7D). In *amy3 bam1* plants, the amount of soluble sugars and proline that accumulated in the root upon osmotic stress was reduced compared with the wild type (Figure 4), whereas sugar and proline accumulation in the leaves was only mildly impaired (Supplemental Figure 7).

Figure 1. (continued).

(B) and **(C)** Leaf starch **(B)** and maltose **(C)** content in osmotically stressed leaves compared with controls. Values are means ± SE ($n = 8$). FW, fresh weight.
(D) Leaf transcript abundance for *BAM1*, *BAM3*, and *AMY3* in osmotically stressed and control leaves, determined by qPCR. Plants grown as above were harvested at the indicated time points. The *ACT2* gene was used as a reference gene. *RD29A* was used as a positive stress-induced control. Values were normalized against gene expression at T0 (set as 1) and represent means ± SE ($n = 3$). Statistical significances determined by unpaired two-tailed Student's *t* tests: *P < 0.05 for the indicated comparison; #P < 0.05 mutants versus the wild type at the indicated time points; n.s., not significant for the indicated comparison.

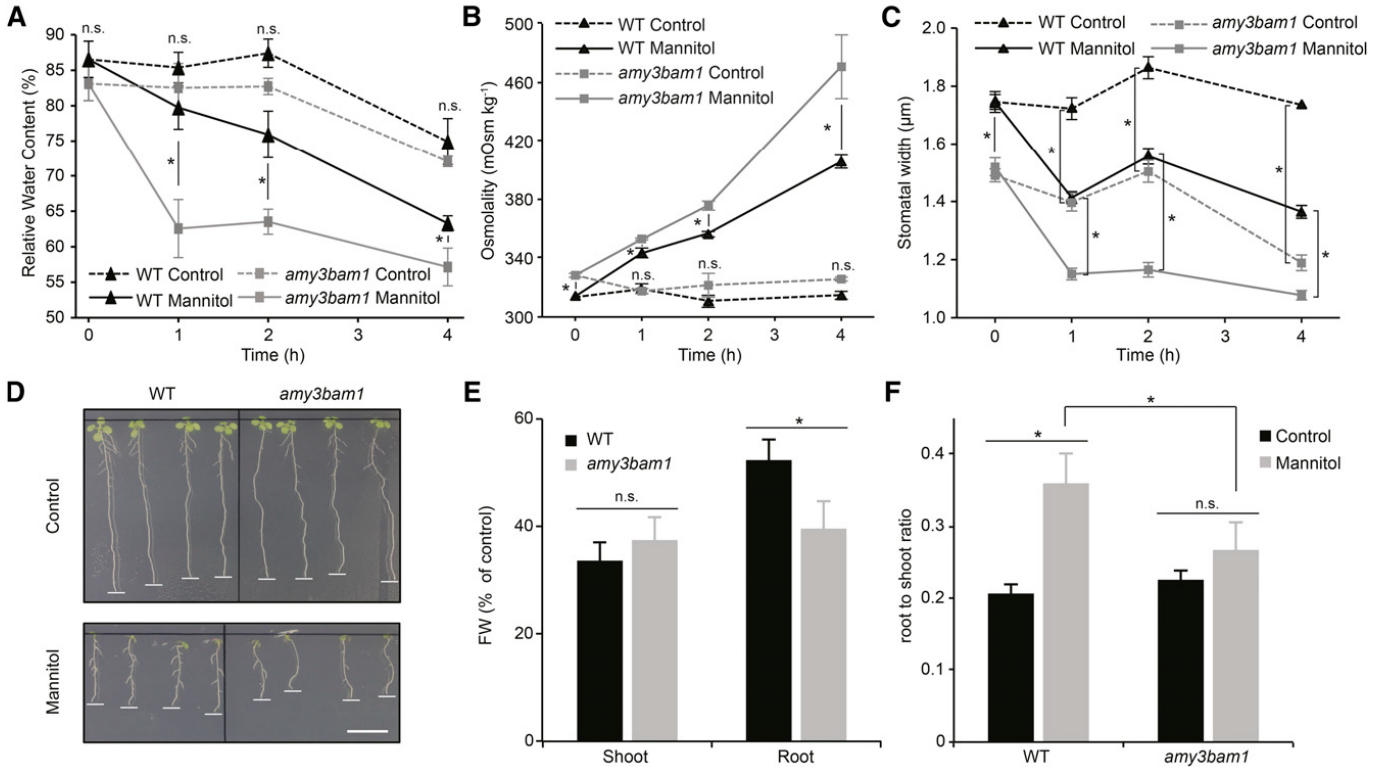

Figure 2. Effects of Osmotic Stress on *amy3 bam1* Double Mutants.

(A) Leaf relative water content of wild-type and *amy3 bam1* plants subject to mannitol stress or kept in control solution was determined at the indicated time points as described in Methods. Values are means ± SE (n = 6).

(B) Osmolality of wild-type and *amy3 bam1* leaf sap. Values are means ± SE (n = 6).

(C) Stomatal closure in response to mannitol treatment in wild type and *amy3 bam1* plants. Epidermal peels isolated from leaves of hydroponically grown plants treated with mannitol for 4 h or kept in a control nutrient solution were used for stomatal width measurements. Values are means ± SE (n = 4 biological replicates with more than 50 individual stomata measured for each time point).

(D) Morphology of wild-type and *amy3 bam1* plants under control (top panel) and osmotic stress conditions (bottom panel). Plants were grown in control medium for 6 d, transferred to medium optionally supplemented with 300 mM mannitol, and photographed 3 d later. Bar = 1 cm.

(E) Shoot and root fresh weights (FW) measured 9 d after seedling transfer as described in **(D)**. Values represent the FW of osmotically stressed plants compared with control plants (set as 100%). Values are means ± SE (n = 15).

(F) Root to shoot ratio of wild-type and *amy3 bam1* plants in response to 300 mM mannitol stress. Values are derived from the data shown in **(E)**. Statistical significances determined by unpaired two-tailed Student's t tests: *$P < 0.05$ for the indicated comparison; n.s., not significant for the indicated comparison.

Thus, osmotic stress seems to lead to (1) carbon accumulating in sugars, both in leaves and roots; (2) increased carbon export to the root; and (3) a reduction in carbon partitioning toward starch and cell wall. The most obvious consequence of defective starch degradation upon osmotic stress in the *amy3 bam1* mutant is a reduction in carbon allocation to the root, which may explain the hypersensitivity of *amy3 bam1* to the stress treatment.

Application of Exogenous ABA Induces *BAM1* and *AMY3* Expression and Results in Increased BAM1 Enzyme Activity and Starch Degradation

Mannitol treatment caused an increase in endogenous ABA levels in the leaves of both wild-type and *amy3 bam1* plants (Supplemental Figure 8). The rapid biosynthesis of ABA is recognized as one of the pivotal events in osmotic stress responses. ABA facilitates stomatal closure and induces the expression of many stress-responsive genes that protect plants from further water loss and damage (Urano et al., 2009; Yamaguchi-Shinozaki and Shinozaki, 2006; Choudhury and Lahiri, 2011; Böhmer and Schroeder, 2011; Zeller et al., 2009; Matsui et al., 2008; Kempa et al., 2008). We wondered whether ABA had an effect on leaf starch metabolism. Exogenously applied ABA (4 h) induced the expression of *BAM1*, *RD29A*, and, to a lesser extent, *AMY3* but had no effect on *BAM3* (Figure 5A), somewhat mirroring the behavior of these genes under osmotic stress treatment (Figure 1D). BAM1 levels and activity already increased in ABA-treated plants relative to untreated plants after 2 h of exposure to ABA (Figures 5B and 5C; Supplemental Figure 9), indicating that transcriptional activation resulted in rapid de novo protein synthesis. In contrast, there was no detectable change in AMY3 protein level in response to ABA (Supplemental Figure 5B). However, *AMY3* transcripts and spectra matching AMY3-derived peptides are frequently detected in many tissues types, especially leaves (Supplemental Figure 5A) (Baerenfaller et al., 2011), suggesting that the protein is already relatively abundant and that it most likely undergoes

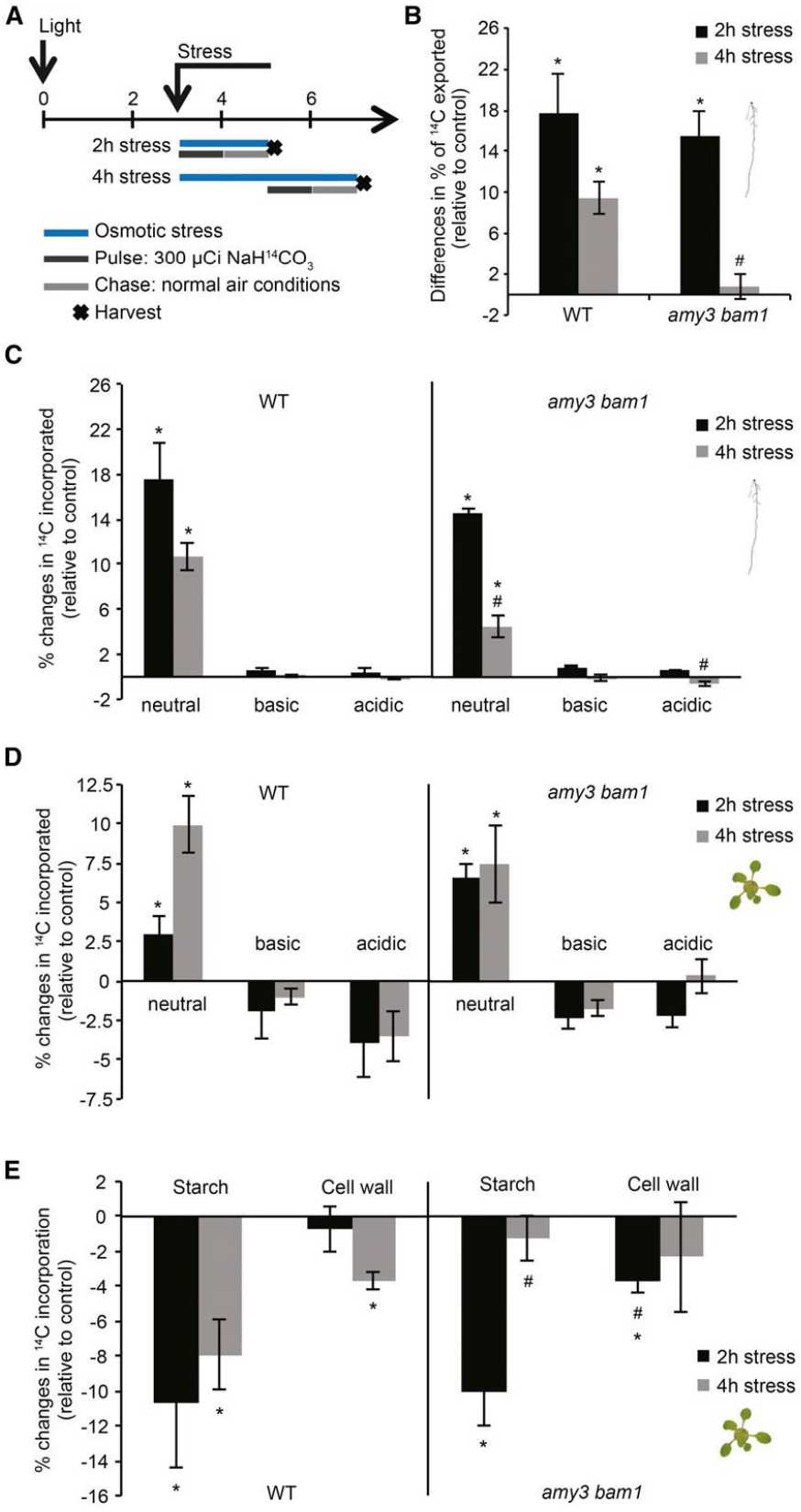

Figure 3. Carbon Partitioning in Wild-Type and *amy3 bam1* Plants during Osmotic Stress.

(A) Scheme of labeling set up. Whole wild-type and *amy3 bam1* plants were labeled with $^{14}CO_2$ for 1 h, just after transfer to a mannitol-containing nutrient solution or at the middle of the stress treatment. Following a 1-h chase period, the shoot and root were harvested separately and the ^{14}C in the different tissue fractions determined by scintillation counting.

posttranslational regulation. Interestingly, ABA-treated plants accumulated less starch than the controls, and maltose levels were high (Figures 5D and 5E). In contrast, ABA-sprayed *amy3 bam1* mutants accumulated similar amounts of starch to untreated plants, and maltose levels were slightly reduced, indicating that starch degradation in response to ABA was not activated in this mutant (Figures 5D and 5E). Despite the differences in stomatal width at the beginning of the ABA treatment (reduced in *amy3 bam1*, as expected), the wild type and *amy3 bam1* reduced their stomatal width to a similar extent (1.13 and 1.18 μm, respectively) within the first hour (Figure 5F). It is possible that during stomatal closure, ABA-regulated *AMY3* and *BAM1* expression might provide a feedback mechanism that controls stomatal width by increasing osmotically active solute concentrations, thereby counteracting the effects of potassium ion efflux. Stomata stayed closed in both genotypes until the end of the experiment (Figure 5F).

Altogether, these results suggest that exogenous ABA can trigger BAM1/AMY3-mediated starch degradation in the leaves and that this effect is independent of ABA-induced stomatal closure.

The ABA-Deficient Mutants *nced3* and *aba2* Mimic the *amy3 bam1* Phenotype under Osmotic Stress

An essential step in stress-induced de novo ABA biosynthesis is the cleavage of epoxycarotenoids to produce xanthoxin (the first C15 intermediate) by the 9-*cis* EPOXYCAROTENOID DIOXYGENASE3 (NCED3) (Iuchi et al., 2001). This explains why the *nced3*-null mutant does not accumulate ABA in response to dehydration (Iuchi et al., 2001; Urano et al., 2009). We used the *nced3* mutant to further investigate the role of ABA in stress-responsive starch metabolism. Under osmotic stress, *nced3* mimicked the behavior of *amy3 bam1*: Starch degradation was abolished and maltose levels remained unchanged compared with control plants, whereas the wild type activated starch degradation, as expected (Figures 6A and 6B). Given the tight link between ABA and sugar signaling pathways (reviewed in Hey et al., 2010), we wondered whether the defective starch degradation in *nced3* under osmotic stress was simply a secondary effect of ABA-sugar crosstalk rather than a direct consequence of the lack of stress-induced de novo ABA biosynthesis. We then investigated the phenotype of other ABA-related mutants. Similar to *nced3*, the ABA-deficient mutant *aba2* fails to synthesize ABA, as it lacks the short-chain alcohol dehydrogenase ABA2 responsible for the conversion of xanthoxin to abscisic aldehyde (González-Guzmán et al., 2002; Schwartz et al., 1997). As observed for *nced3*, *aba2* also failed to activate starch degradation and showed no or much reduced maltose accumulation in response to osmotic stress (Supplemental Figure 10). In contrast, the *aao3* mutant, lacking the ABSCISIC ALDEHYDE OXIDASE3 (AAO3) responsible for the final step in ABA biosynthesis (Seo and Koshiba, 2002), activated starch degradation similarly to the wild type (Figures 6A and 6B). Given that Arabidopsis contains four *AAO* genes with partially redundant functions (Seo et al., 2004), *aao3* mutants accumulate reduced but still detectable levels of ABA in response to dehydration stress (Seo et al., 2000), which may account for the activation of starch degradation in this mutant. Furthermore, *BAM1*, as well as *RD29A*, were significantly upregulated in response to stress in *aao3*, although to a lesser extent compared with the wild type, but were not induced in *nced3* (Figure 6C). *AMY3* was upregulated in the wild type, but not in *aao3* or *nced3* (Figure 6C), substantiating the importance of posttranslational regulation for the activation of AMY3 under stress. As expected, *BAM3* gene expression remained unaltered in all tested genotypes (Figure 6C). Together, these results show that the differences in ABA levels between the wild type and *aao3*, *nced3*, and *aba2* mutants are likely to be responsible for the observed differences in stress-induced starch degradation, suggesting that de novo ABA biosynthesis in response to osmotic stress is required for starch degradation. Consistently, exogenous application of ABA alone or in combination with mannitol restored starch degradation in the *nced3* mutant to almost wild-type levels (Supplemental Figure 11).

The AREB/ABF-SnRK2 Pathway Regulates ABA-Dependent *BAM1* and *AMY3* Gene Expression

To understand the molecular link between ABA and starch degradation during osmotic stress, we searched the promoter regions of *BAM1* and *AMY3* genes for known ABA-dependent *cis*-regulatory elements. Both *BAM1* and *AMY3* promoters contained ABA-responsive element (ABRE) motifs, while none were present in the *BAM3* promoter (Supplemental Data Set 1). The ABRE *cis*-regulatory elements are recognized by a group of bZIP transcription factors (TFs), the ABRE binding protein/ABRE binding factors (AREB/ABFs). These TFs have pivotal functions in ABA-dependent osmotic stress-responsive gene expression (Yoshida et al., 2010). In the presence of ABA, the AREB/ABF TFs are activated through phosphorylation by

Figure 3. (continued).

(B) Carbon export to the roots of osmotically stressed and control plants. Relative changes in ^{14}C imported into the root upon osmotic stress are given as percentages of that imported under control conditions (set as 0). Values are means ± SE ($n = 4$).

(C) Incorporation of ^{14}C into the different water-soluble fractions of wild-type and *amy3 bam1* roots. Relative changes in the amount of ^{14}C incorporated into the different fractions upon osmotic stress are given as percentages of that in corresponding fractions under control conditions (set as 0). Values are means ± SE ($n = 4$).

(D) Incorporation of ^{14}C into the different water-soluble fractions of wild-type and *amy3 bam1* shoots in plants subject to osmotic stress compared with controls. Relative changes are expressed as described above for **(C)**.

(E) Incorporation of ^{14}C into starch and cell wall compounds of wild-type and *amy3 bam1* shoots in plants subject to osmotic stress compared with control. Relative changes are expressed as described above for **(C)**. Statistical significances determined by unpaired two-tailed Student's *t* tests: *$P < 0.05$ for the indicated comparison; #$P < 0.05$ mutant versus the wild type at the indicated time points; n.s., not significant.

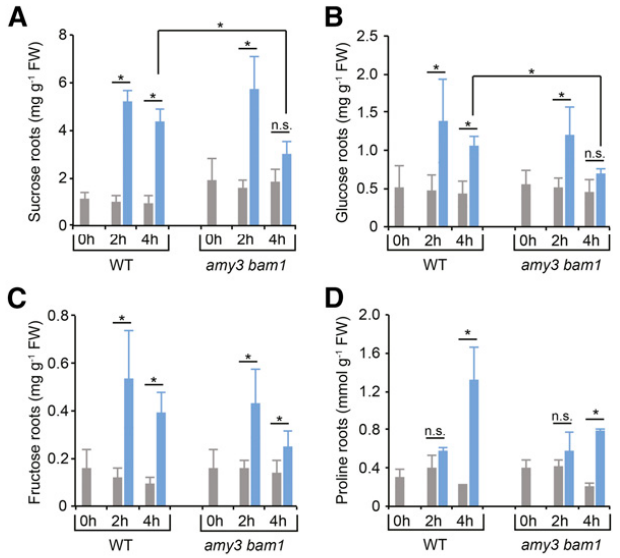

Figure 4. Quantification of Soluble Sugars and Proline in Roots of Osmotically Stressed Plants.

Sucrose **(A)**, glucose **(B)**, fructose **(C)**, and proline **(D)** content in roots of wild-type and *amy3 bam1* plants in response to osmotic stress treatment. Hydroponically grown plants were optionally supplemented with 300 mM mannitol for 4 h. Value are means ± SE ($n = 5$). FW, fresh weight. Statistical significances determined by unpaired two-tailed Student's *t* tests: *P < 0.05 for the indicated comparison; n.s., not significant for the indicated comparison.

SNF1-related kinase 2s (SnRK2s) (Fujita et al., 2009; Furihata et al., 2006), enabling them to bind the ABRE motifs in the promoters of the target genes, thereby activating their expression (Yoshida et al., 2014; Fujita et al., 2013).

The transcriptional activation of *BAM1* and *AMY3* in response to exogenously applied ABA was abolished in mutants lacking three SnRK2 kinases, *snrk2.2 snrk2.3 snrk2.6*, or greatly reduced in mutants lacking three AREB/ABF TFs, *areb1 areb2 abf3* (Figure 7A), all known positive regulators of ABA signaling in the leaves (Nakashima et al., 2009; Umezawa et al., 2009; Fujita et al., 2009; Fujii and Zhu, 2009; Yoshida et al., 2010). The residual transcriptional activation of *BAM1* in the *areb1 areb2 abf3* mutant was most likely due to the remaining activity of another bZIP TF, ABF1, which is also part of the AREB/ABF-Snrk2 signaling pathways in leaves under osmotic stress (Yoshida et al., 2015). Under control conditions, *BAM1* and *AMY3* transcript levels were similar to the wild type in both *snrk2.2 snrk2.3 snrk2.6* and *areb1 areb2 abf3* mutants (Supplemental Figure 12). *BAM3* transcript levels remained unaltered in response to ABA treatment in both mutants, similarly to the wild type (Figure 7A), consistent with the idea that BAM3 induction is not part of the ABA/osmotic stress responses. The *snrk2.2 snrk2.3 snrk2.6* mutant showed a severe wilting phenotype (Fujii and Zhu, 2009), which prevented its successful cultivation in hydroponics. We therefore used the *areb1 areb2 abf3* mutant for further experiments and found that BAM1 protein levels did not change in response to exogenous application of ABA (Figures 7B and 7C), and starch degradation upon mannitol treatment was significantly reduced compared with the wild type (Figures 7D and 7E). Collectively, these findings suggest that ABA-dependent SnRK2 signaling mediates BAM1 and AMY3 activation in response to osmotic stress through AREB1, AREB2, ABF3, and, possibly, ABF1 TFs.

ABREs Are Conserved in the Promoters of *BAM1* Orthologs

In Arabidopsis, the presence of ABREs in the *BAM1* promoter clearly distinguishes BAM1 from BAM3. To determine if this is a general feature of β-amylases from flowering plants, we scanned the promoters of *BAM1* and *BAM3* orthologs from 30 different angiosperm species for the presence of conserved sequence motifs, using the MEME algorithm (Bailey et al., 2009). MEME revealed a collection of conserved motifs across the *BAM1* ortholog promoters, the most highly represented of which was only found in the Eudicots and had a sequence logo CACGTGTC, with a highly significant E-value of 3.6e-49 (Figure 8A; Supplemental Data Set 1). This sequence almost precisely matched the ABRE motif (PyACGTGG/TC) (Zhang et al., 2005; Gómez-Porras et al., 2007), indicating that ABRE *cis*-acting elements are conserved in *BAM1* orthologs from all the eudicotyledon species analyzed here. The distance of the ABREs to the translational start codon (ATG) was also conserved. They were mostly found in the proximity of the ATG in the interval 100 to 400 bp (Figure 8B), consistent with the occurrence of ABREs previously reported for the Arabidopsis genome (Gómez-Porras et al., 2007).

A second motif conserved in all of the analyzed BAM1 orthologs promoters had the sequence logo CA/GCCG/AT/CCC (Figure 8C; Supplemental Data Set 1), which resembled a coupling element 3 (CE3)-like motif (Zhang et al., 2005; Gómez-Porras et al., 2007). CE3-like motifs are found in the promoters of many ABA-responsive genes, often coupled to ABREs. A combination of ABRE/CE3-like motifs was initially thought to establish a minimal ABA-responsive complex to confer ABA responsiveness (Shen and Ho, 1995; Shen et al., 1996). In fact, it was later shown that the ACGT-containing ABREs and CE3 are functionally equivalent *cis*-acting elements and that multiple ABREs or CEs or the combination of ABREs with CEs can also confer ABA responsiveness (Hobo et al., 1999). In our analysis, it is interesting that while ABREs elements were practically absent in the promoters of *BAM1* orthologs from monocotyledons, the CE3-like motifs were universally present (Figure 8B). It is possible that BAM1 orthologs from monocotyledons can be induced by ABA in the absence of ABRE elements, for example, through multiple CE motifs. This hypothesis is consistent with a recent study showing that in rice (*Oryza sativa*), CE3 motifs are overrepresented and ~80 times more frequent than in Arabidopsis (Gómez-Porras et al., 2007). In the promoters of *BAM3* orthologs genes, the MEME algorithm retrieved conserved sequences which could not be assigned to any known regulatory motif (Supplemental Data Set 1). Altogether, this analysis suggests that the presence of ABA responsive elements is a conserved feature of *BAM1* orthologs genes.

DISCUSSION

A Critical Role for Starch Degradation in Osmotic Stress Tolerance

Crops and other plants in natural conditions are routinely affected by a broad range of abiotic and biotic stresses acting simultaneously or in sequence. This results in a high degree of complexity

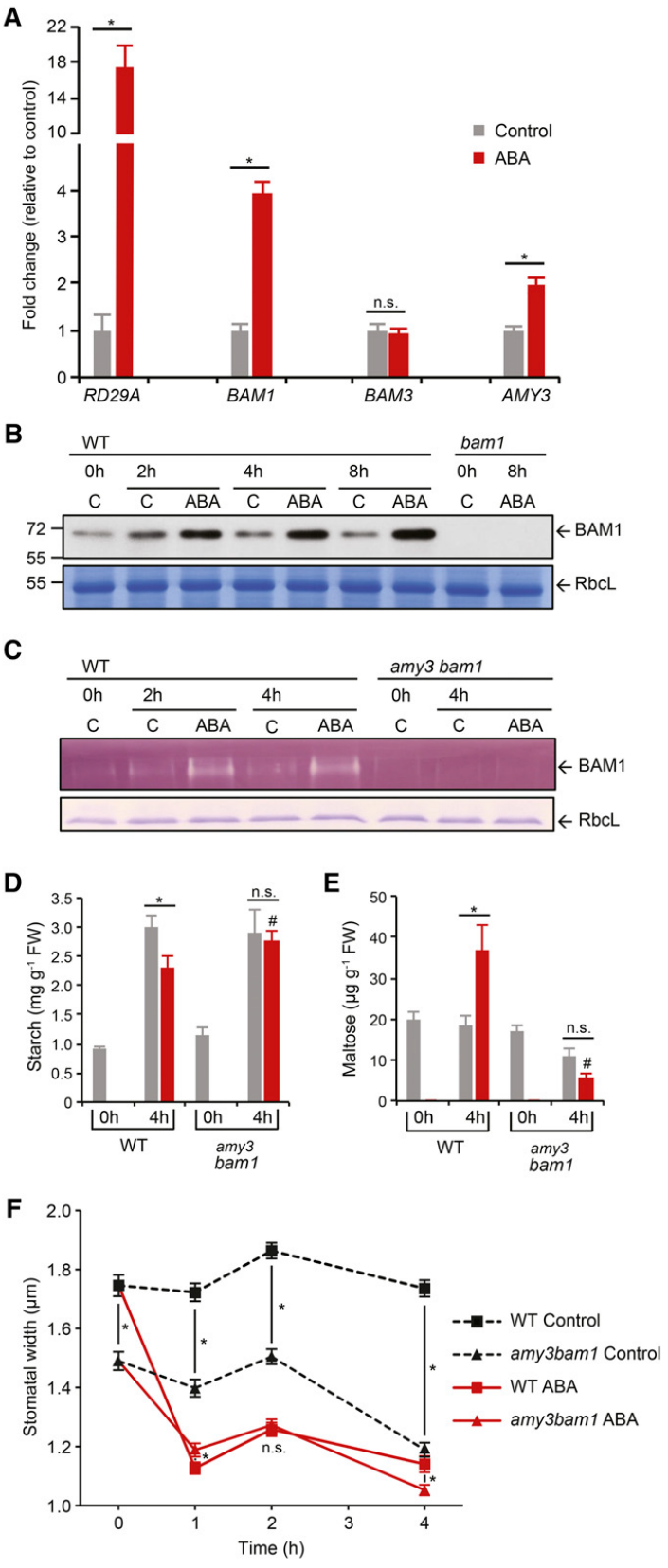

Figure 5. Effects of Exogenous ABA on Leaf Starch Metabolism.

(A) Relative expression levels of *BAM1*, *BAM3*, and *AMY3* in wild type leaves 4 h after treatment with 100 µM ABA, determined by qPCR. The *ACT2* gene served as a reference gene. *RD29A* served as a positive control for the ABA treatment. Values representing means ± SE ($n = 3$) were normalized against gene expression in control conditions (set as 1).

(B) Immunodetection of BAM1 protein in wild-type leaves after ABA treatment. Total protein was extracted from rosettes of hydroponically grown plants at the indicated time points. Equal protein amounts were separated by SDS-PAGE. The Rubisco large subunit (RbcL), the dominant band visualized by Coomassie staining, confirmed uniform loading. BAM1 was detected using polyclonal antibodies raised against recombinant BAM1. Extracts of the *bam1* mutant served as a negative control. Replicate blots yielded the same result. C, mock-treated control.

(C) ABA-mediated changes in BAM1 activity. Leaf crude extracts from hydroponically grown wild-type and *amy3 bam1* plants harvested 4 h after ABA treatment were separated by native PAGE in gels containing 0.1% amylopectin. After electrophoresis and incubation for 2 h (see Methods), the gels were stained in Lugol's solution. BAM1 activity was detected in wild-type but not *amy3 bam1* plants.

(D) and **(E)** Leaf starch **(D)** and maltose **(E)** content in wild-type and *amy3 bam1* plants 4 h after ABA treatment, compared with controls. Values are means ± SE ($n = 6$). FW, fresh weight.

(F) Stomatal closure in response to ABA treatment in wild-type and *amy3 bam1* plants. Epidermal peels isolated from leaves of hydroponically grown plants treated with ABA 100 µM for 4 h or kept in a control nutrient solution were used for stomatal width measurements. Values are means ± SE (n = 4 biological replicates with more than 50 individual stomata measured for each time point). Stomatal width under control conditions is the same as in Figure 2C, as the experiments were conducted in parallel. Statistical significances determined by unpaired two-tailed Student's *t* tests: *$P < 0.05$ for the indicated comparison; #$P < 0.05$ mutant versus the wild type at the indicated time points; n.s., not significant for the indicated comparison.

in plant stress responses in the field, making it often difficult to dissect the single components. In this study, the use of hydroponic and agar plate systems enabled us to investigate the effects of osmotic stress on both leaves and roots and define the role of stress-induced starch degradation on the metabolism of the plant as a whole. Even though the field environment is very different from the controlled conditions used in the laboratory, our discovery uncovers a critical function for starch in plant osmotic stress tolerance and highlights two starch hydrolases, AMY3 and BAM1, as targets for future studies under field conditions.

We provide evidence that the reduction in starch accumulation in Arabidopsis plants exposed to a short-term, high osmotic stress is, at least in part, a result of induced starch degradation, leading to the production of maltose, the major starch catabolite (Figures 1B and 1C). In mutants lacking AMY3 and BAM1, this metabolic stress response was abolished and starch accumulation was the same as in control conditions (Figures 1B and 1C). Furthermore, the maltose levels in the starch-less Arabidopsis mutant *pgm* remained unchanged in response to osmotic stress (Supplemental Figure 2). Concomitantly with the induction of starch degradation, wild-type plants responded to the osmotic stress by accumulating high levels of sugars and proline in the leaves (Supplemental Figure 7), presumably for osmotic adjustment and energy supply to maintain cell survival and metabolic activity (Couée et al., 2006; Verslues and Sharma, 2011). Our data suggest that most of these osmolytes derived from photosynthetic carbon assimilation and only partly from starch hydrolysis. First, *amy3 bam1* plants were only mildly impaired in leaf sugar accumulation during stress and showed no differences from the

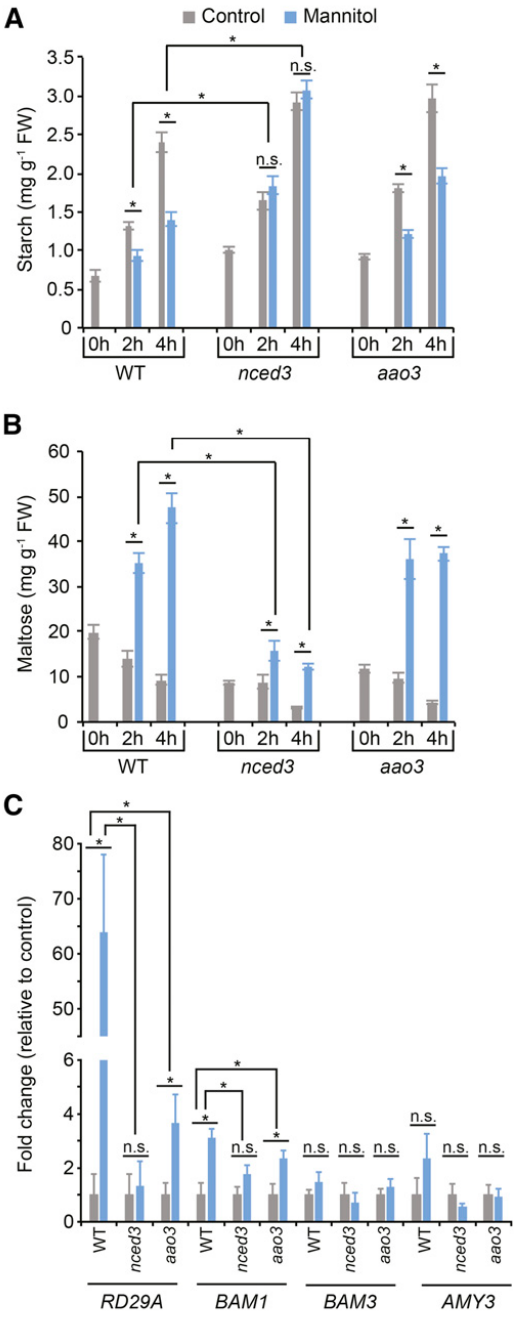

Figure 6. Leaf Starch Degradation during Osmotic Stress Is Blocked in the ABA-Deficient Mutant *nced3*.

(A) and **(B)** Leaf starch **(A)** and maltose **(B)** content in wild-type, *nced3*, and *aao3* plants treated with 300 mM mannitol compared with controls. Values are means ± SE (n = 6). FW, fresh weight.

(C) Relative expression levels of *BAM1*, *BAM3*, and *AMY3* in leaves of wild-type, *nced3*, and *aao3* plants treated with 300 mM mannitol for 4 h, determined by qPCR. The *ACT2* gene served as a reference gene. *RD29A* served as a positive control for the osmotic stress treatment. Values representing means ± SE (n = 3) were normalized against gene expression in control conditions (set as 1). Statistical significances determined by unpaired two-tailed Student's t tests: *$P < 0.05$ for the indicated comparison; n.s., not significant for the indicated comparison.

wild type in leaf proline accumulation (Supplemental Figure 7). Moreover, our ^{14}C labeling experiments showed that carbon partitioning into the leaf soluble neutral fraction during stress was similar in wild-type and *amy3 bam1* plants (Figure 3D). Consistent with this, we observed that osmotic stress caused only a transient decrease in the PSII operating efficiency (ΦPSII), which was similar in the two genotypes (Supplemental Table 3) and, importantly, did not hinder the capacity of the plants to assimilate $^{14}CO_2$. However, sugars may also accumulate in leaves because of decreased demand, as a consequence of shoot growth limitation (Hummel et al., 2010), or because of decreased starch biosynthesis (Geigenberger et al., 1997). This might be caused by changes in phosphorylated intermediates, especially 3-phosphoglycerate, reduction of which would inactivate ADP-glucose pyrophosphorylase, the regulated enzyme of starch biosynthesis (Heldt et al., 1977). A decline in 3-phosphoglycerate under stress could result from impaired photosynthesis (Kaplan and Guy, 2005; Scarpeci and Valle, 2008) or from the activation of the sucrose biosynthesis in response to stress through sucrose synthase phosphate (Geigenberger et al., 1997), which would compete for assimilates. It was indeed demonstrated that phosphorylation of sucrose synthase phosphate is a mechanism for osmotic stress activation of this enzyme in spinach leaves (Toroser and Huber, 1997).

Major differences between the wild type and *amy3 bam1* were apparent in the amount of carbon channeled to the root under osmotic stress. This was markedly reduced in the mutant compared with the wild type, especially after 4 h of mannitol treatment (Figure 3B). Consequently, *amy3 bam1* had reduced levels of sugars and proline in the roots (Figure 4). It is possible that starch degradation in the leaves is required to increase carbon exported to the roots during osmotic stress (Figure 9). However, it cannot be excluded that *amy3 bam1* suffered additional defects, such as impaired phloem loading and long distance sugar transport, which would contribute to the complexity of the observed phenotype. Reduced carbon export in *amy3 bam1* most likely affected the ability of the root to absorb water and nutrients from the medium (Supplemental Figure 6; Figure 9). An early study reported that inorganic ion uptake regulates turgor in osmotically stressed Arabidopsis epidermal root cells (Shabala and Lew, 2002). Thus, carbon export to the root during osmotic stress could provide the energy needed for this uptake, besides directly contributing to the root osmotic adjustment.

There was also an impact on root growth. While wild-type plants responded to osmotic stress by increasing their root to shoot ratio, the ratio in *amy3 bam1* remained unchanged compared with non-stress conditions (Figure 2F). Adjustment of root growth under stress is an important survival strategy, allowing plants to expand the root (at the expense of shoot growth) to increase nutrient and water uptake capacity (Wu and Cosgrove, 2000; Rogers and Benfey, 2015; Roycewicz and Malamy, 2012). Our results are consistent with these observations and reveal a function of starch degradation in controlling root growth in response to osmotic stress (Figure 9).

Differential Regulation and Isoform Subfunctionalization Define the Adaptive Plasticity of Plant Starch Metabolism

The two close homologs, BAM1 and BAM3, are differentially regulated in response to abiotic stresses (Figure 1D; Supplemental Figure 13) (Maruyama et al., 2009; Kaplan and Guy, 2004; Monroe

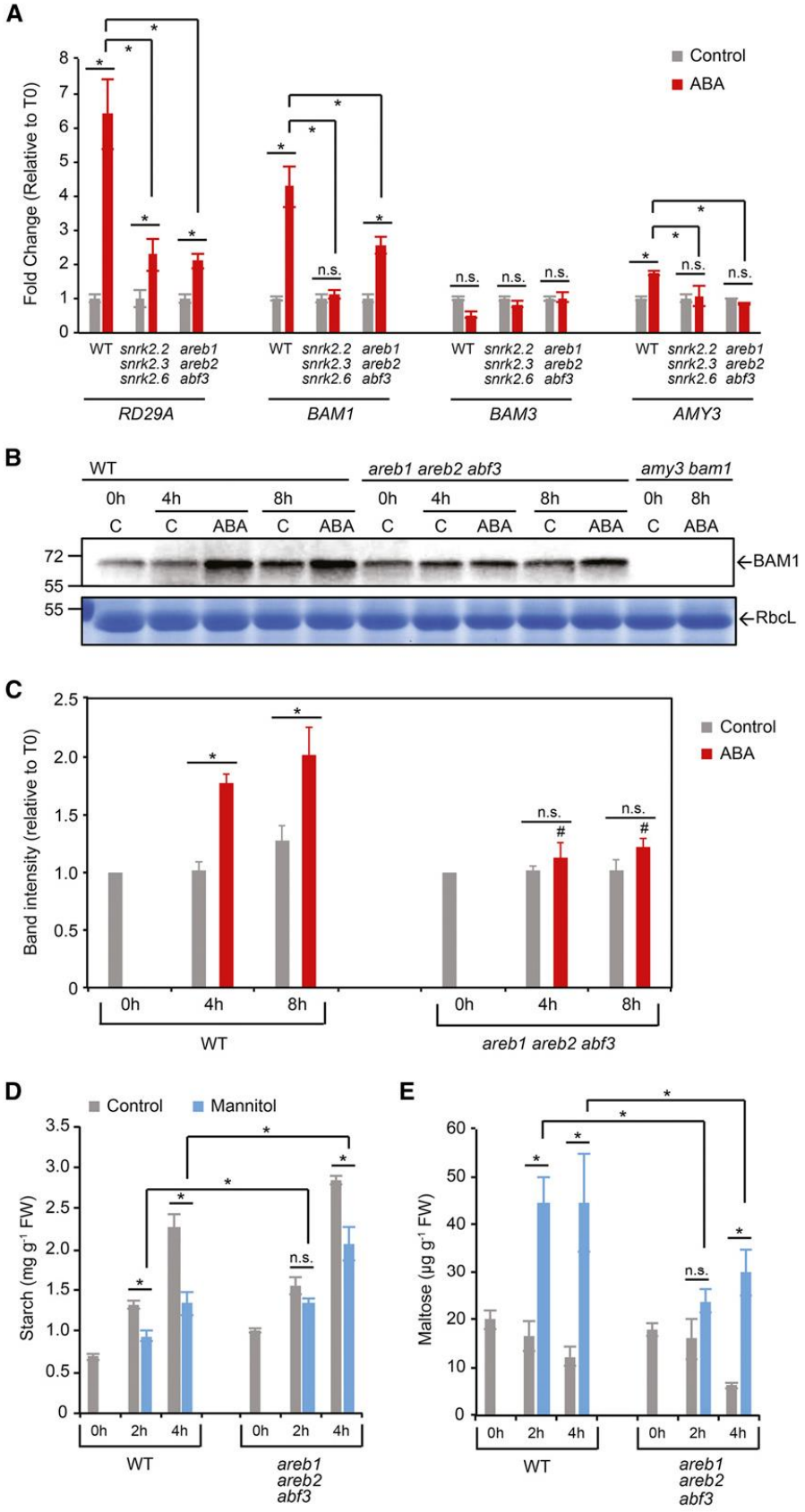

Figure 7. AREB1, AREB2, and ABF3 Transcription Factors Regulate *BAM1* and *AMY3* Expression in Response to Osmotic Stress.

(A) Relative expression levels of *BAM1*, *BAM3*, and *AMY3* in the wild type and *areb1 areb2 abf3* triple mutant leaves 4 h after treatment with 100 µM ABA, determined by qPCR. The *ACT2* gene served as a reference gene. *RD29A* served as a positive control for the ABA treatment. Values representing means ± SE (n = 3) were normalized against gene expression in control conditions (set as 1).

(B) Immunodetection of BAM1 protein in wild-type, *areb1 areb2 abf3*, and *amy3 bam1* leaves after ABA treatment. Total protein was extracted from rosettes of hydroponically grown plants at the indicated time points. Equal amounts of protein were separated by SDS-PAGE and the Rubisco large subunit (RbcL)

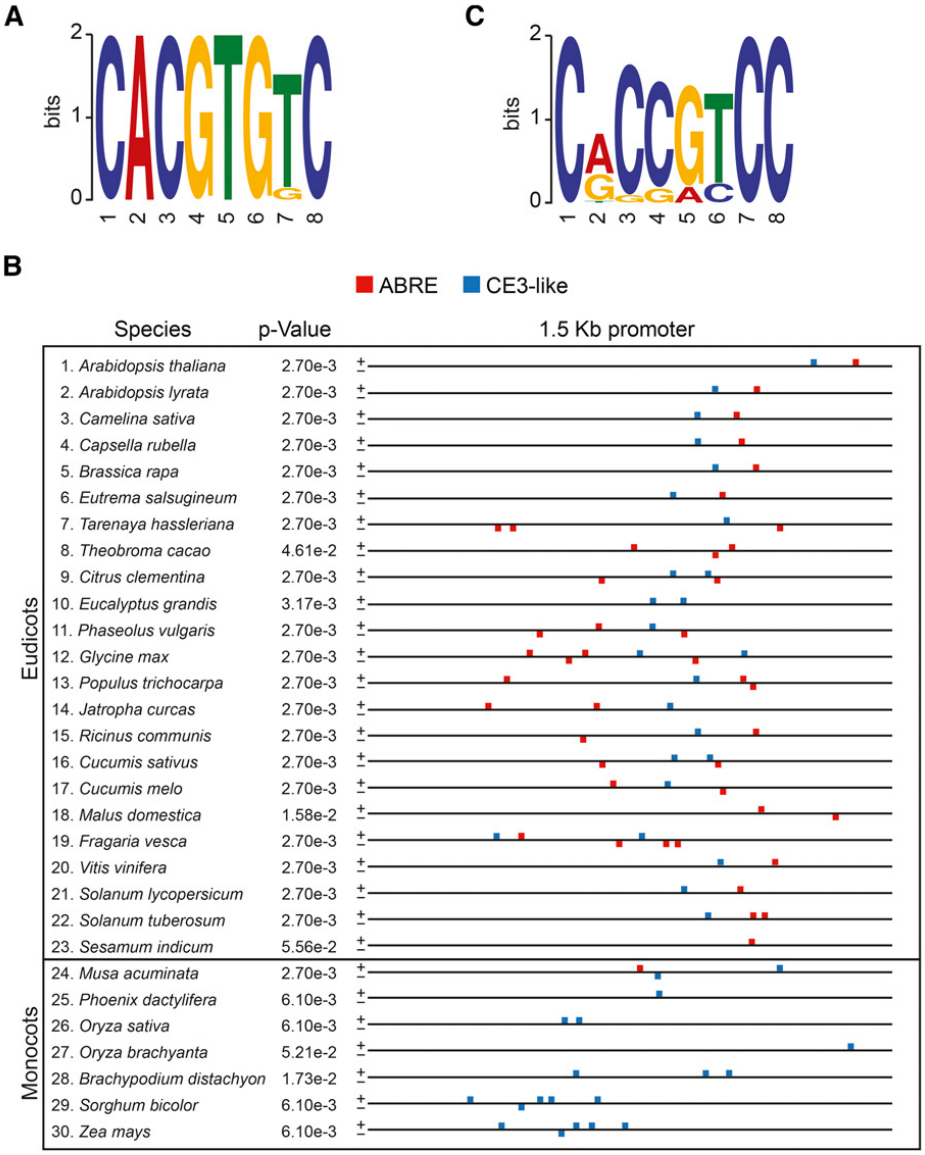

Figure 8. Unbiased Bioinformatics Analysis of *BAM1* Ortholog Promoter Sequences.

(A) and **(B)** The 1.5-kb promoter regions of *BAM1-like* genes from 30 angiosperm species were analyzed using the MEME algorithm (http://meme.nbcr.net). The conserved sequence logos of the ABREs and CE3-like found by MEME are depicted in **(A)** and **(B)**, respectively. The diagram provides an idea of which positions in the motif are most highly conserved (measured in bits). Highly conserved positions in the motif have higher bits.
(C) Distribution of ABRE and CE3-like *cis*-regulatory elements within the analyzed promoters.

et al., 2014). *BAM3* gene expression is induced by cold stress, whereas *BAM1* expression is induced by heat and by osmotic stress. The most obvious interpretation of this differential transcriptional regulation is that starch degradation can be induced by different signals. Mutant studies substantiate the hypothesis that *BAM1* induction is critically important in the osmotic stress response. As first reported by Valerio et al. (2011), we also found that *bam1* mutants had reduced starch degradation in response to

Figure 7. (continued).

was used for confirmation. BAM1 was detected using polyclonal antibody raised against recombinant BAM1. Replicate blots yielded the same result. C, control.
(C) BAM1 protein quantification. Densitometry analysis (ImageJ) was used to quantify band intensities such as in **(B)**. Values are means ± SE of three biological samples, each analyzed with three technical replicates, and expressed relative to the mean band intensity at time 0 (T0, set as 1).
(D) and **(E)** Starch **(D)** and maltose **(E)** content in wild-type and *areb1 areb2 abf3* plants subject to mannitol stress compared with controls. Values are means ± SE ($n = 6$). FW, fresh weight. Statistical significances determined by unpaired two-tailed Student's *t* tests: *$P < 0.05$ for the indicated comparison; #$P < 0.05$ mutant versus the wild type at the indicated time points; n.s., not significant for the indicated comparison.

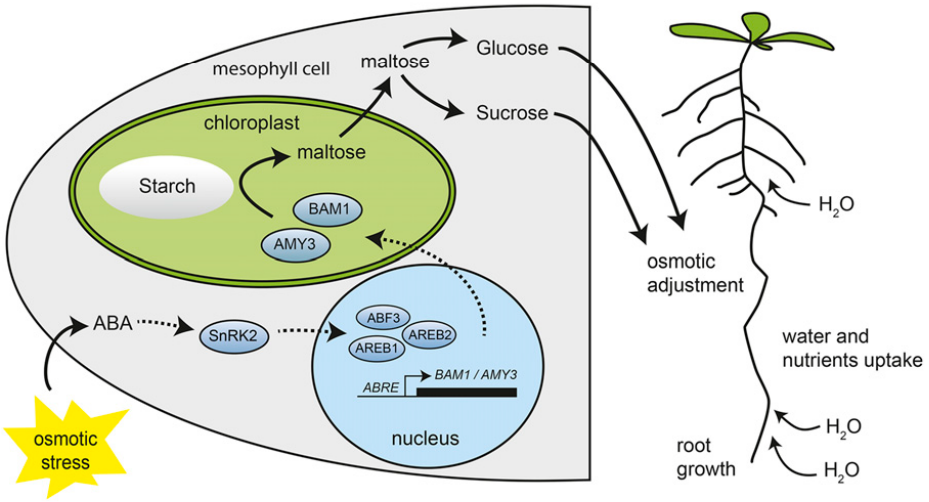

Figure 9. Proposed Model of Starch Degradation Mechanism and Regulation during Osmotic Stress.

In response to stress, ABA triggers *BAM1* and *AMY3* transcription through the ABA-dependent AREB/ABF-SnRK2 signaling pathway. This leads to rapid de novo BAM1 protein synthesis and increased amylolytic activity, although posttranslational modifications are also likely to contribute. A fraction of the maltose released from starch by the synergistic action of BAM1 and AMY3 is exported to the cytosol and metabolized into sucrose and free hexoses. Sucrose is then exported to the root to support osmotic adjustment, water and nutrient uptake, and root growth. The remaining sugars, including some maltose and the additional sugars originating from carbon assimilation, are retained in the leaves for osmotic adjustment, energy supply, and to protect the photosynthetic apparatus from oxidative stress.

short-term mannitol treatment (Figures 1B and 1C). In contrast, *bam3* mutants, though compromised in normal nighttime starch degradation (Supplemental Figure 3), could activate *BAM1* expression and starch degradation under osmotic stress conditions (Figures 1B and 1C; Supplemental Figure 4), showing that BAM3 is not part of the osmotic stressed-induced starch degradation pathway. A similar situation was found in guard cells. Under standard growth conditions, *BAM1* is preferentially and highly expressed in guard cells, whereas *BAM3* is considerably less abundant (Horrer et al., 2016). Consistent with the gene expression pattern, *bam1* mutants have elevated guard cell starch levels compared with the wild type, whereas *bam3* accumulates starch in this cell type similarly to the wild type (Horrer et al., 2016; Valerio et al., 2011). Altogether, these findings indicate that BAM1 and BAM3 are active under different conditions and in a cell type-specific manner. Subfunctionalization among members of the β-amylase family has occurred that significantly contributed to the ability of the plant to adjust starch turnover to the need of the individual cells and in response to the external environmental stimuli (Monroe et al., 2014; Zanella et al., 2016; Horrer et al., 2016).

Besides subfunctionalization, synergy among the enzymes of starch degradation is also critical in defining starch adaptive plasticity. We recently showed that BAM1 and AMY3 work synergistically in guard cells to degrade starch during stomatal opening (Horrer et al., 2016). Here, we report that AMY3 is also involved with BAM1 in mediating starch breakdown in the leaves in response to osmotic stress. It is conspicuous that the loss of both enzymes does not affect starch metabolism under normal conditions (Supplemental Figure 3), yet completely blocks starch degradation in guard cells (Horrer et al., 2016) or in the leaves upon osmotic stress (Figures 1B and 1C). These contrasting phenotypes hint at an intricate network of differential regulation which defines subfunctionalization among the enzymes of starch degradation, with some required for the mobilization of starch to respond to stress, and others required for normal nighttime starch degradation in leaves or for daytime starch degradation in guard cells.

Previous work suggests that it may not be just the gene expression patterns, but also the characteristics of encoded BAM1 and AMY3 enzymes themselves, that distinguish them from other starch-degrading enzymes and make them suitable for starch degradation during daytime. For example, both enzymes are redox regulated, whereby they can be rapidly activated through reduction via the light-driven ferredoxin-thioredoxin system (Sparla et al., 2006; Seung et al., 2013). Furthermore, both enzymes are preferentially active at a slightly alkaline pH (Seung et al., 2013; Sparla et al., 2006; Monroe et al., 2014; Santelia et al., 2015). Thus, the reducing environment and the alkaline pH of the stroma, generated by the photosynthetic electron transport chain in the light (Heldt et al., 1973; Buchanan and Balmer, 2005), would favor BAM1 and AMY3 activity. Conversely, there is no evidence for redox regulation of BAM3, and the enzyme has optimum activity at less alkaline pH (Santelia et al., 2015; Monroe et al., 2014). Furthermore, large-scale site-specific phosphorylation profiling of Arabidopsis proteins revealed the presence of one or more phosphorylation sites on both BAM1 and AMY3 proteins (de la Fuente van Bentem et al., 2008; Reiland et al., 2009; Xue et al., 2013; Heazlewood et al., 2008). Given the rapid activation of leaf starch degradation in response to the osmotic stress, it is likely that posttranslational modifications such as protein phosphorylation or redox regulation played a major role in activating BAM1 and AMY3 in the light. This would be particularly crucial for AMY3, as the protein was already relative abundant in the leaves, and no changes in protein levels were detected upon treatment (Supplemental Figure 5).

Lastly, it should be noted that there are likely to be factors required for starch degradation in osmotic stress conditions other than BAM1 and AMY3. For example, the enzymes mediating the phosphorylation and dephosphorylation of starch, a process that precedes its degradation by amylases, may be required for osmotic stress-induced degradation as well as for normal nighttime degradation (Yano et al., 2005; Silver et al., 2014; Kötting et al., 2010). However, GWD, which phosphorylates the C6-position of glucosyl residues (Ritte et al., 2006), was reported to be involved in the cold-induced development of freezing tolerance (Yano et al., 2005), similar to BAM3 (Kaplan and Guy, 2004). Thus, it is plausible that a certain degree of subfunctionalization also exists within the enzymes of glucan phosphorylation, with GWD required for the mobilization of starch under cold stress, and PWD, which phosphorylates the C3 positions, required for the activation of starch degradation under osmotic stress.

ABA Is the Primary Signal for Starch Degradation in Leaves in Response to Osmotic Stress

One of the pivotal events in osmotic stress responses is the rapid, transient accumulation of ABA, which facilitates stomatal closure and expression of ABA-responsive genes that protect plants from further water loss and damage. Several transcriptomic analyses have reported ABA-dependent induction of a multitude of dehydration stress-related genes, as well as some involved in primary carbohydrate metabolism (Böhmer and Schroeder, 2011; Kempa et al., 2008; Matsui et al., 2008; Choudhury and Lahiri, 2011; Urano et al., 2009; Fujita et al., 2011). Here, we showed that BAM1 and AMY3 are targets of the ABA/osmotic-dependent AREB/ABF-SnRK2 pathway and that ABA is required for osmotic stress-induced starch degradation in the leaves (Figure 9). Exogenously applied ABA induced *BAM1* and *AMY3* expression in the wild type, but not in mutants lacking key components of the leaf ABA/osmotic-dependent signaling pathway (Figure 7A) (Yoshida et al., 2014; Fujii and Zhu, 2009). Consistently, exogenously applied ABA stimulated starch degradation in the wild type, but not in the double mutant *amy3 bam1* (Figures 5D and 5E), and this effect appeared to be independent of ABA-induced stomatal closure (Figure 5F). Thus, activation of BAM1 and AMY3 in response to ABA (Figures 5B and 5C; Supplemental Figure 5) induces starch degradation. Lastly, osmotic stress-induced starch degradation was abolished in the *nced3* and *aba2* mutants, but not in a mutant lacking AAO3 (Figures 6A and 6B; Supplemental Figure 10). Given that the major difference between these ABA biosynthetic mutants is their capacity to accumulate ABA in response to stress (Seo et al., 2000; Iuchi et al., 2001; Urano et al., 2009), our data suggest that the lack of stress-induced starch degradation in *nced3* and *aba2* was specifically caused by the complete absence of ABA accumulation. This is supported by the observation that the stress-induced transcriptional induction of *BAM1* and *AMY3* was substantially impaired in *nced3*, but not in *aao3* (Figure 6C). These observations are in line with previous transcriptomic and metabolic data and lead to the conclusion that de novo biosynthesis of ABA is the trigger for starch degradation in the light in response to osmotic stress.

Both *BAM1* and *AMY3* promoters contain ABRE motifs, which are absent in the *BAM3* promoter (Supplemental Data Set 1), consistent with the observation that *BAM3* is not ABA-induced. Pairs of BAM1 and BAM3 orthologs are found in the genomes of several angiosperms, suggesting that BAM isoform subfunctionalization is a general feature of flowering plants (Monroe et al., 2014; Fulton et al., 2008). Interestingly, bioinformatics analyses of the promoters of *BAM1* and *BAM3* orthologs from 30 different plant species using the MEME algorithm (Bailey et al., 2009) revealed an enrichment of ABRE elements in the proximity of the ATG codon in all examined *BAM1-like* gene promoters, with the exception of that of the grasses, where instead CE3-like ABA-responsive elements were found (Figure 8; Supplemental Data Set 1). Furthermore, ABRE or CE3 were absent in all examined *BAM3-like* gene promoters (Supplemental Data Set 1). This suggests that the presence of ABA-responsive elements is a conserved feature of *BAM1* orthologs and that the regulation of starch degradation by ABA during stress through the AREB/ABF-SnRK2 pathway is most likely a conserved mechanism across many plant species.

METHODS

Plant Materials and Growth Conditions

The following *Arabidopsis thaliana* T-DNA insertion mutants were used in this study: *bam1* (Salk_039895), *bam3* (CS92461) (Fulton et al., 2008), *amy3-2* (Sail_613 D12) (Yu et al., 2005), *amy3 bam1* (Horrer et al., 2016), *snrk2.2 snrk2.3 snrk2.6* (Fujii and Zhu, 2009), *areb1 areb2 abf3* (Yoshida et al., 2010), *aao3-4* (Salk_072361) (Seo et al., 2004), *nced3* (GABI_129B08) (Wan and Li, 2006), and *aba2-1* (G1464A) (Léon-Kloosterziel et al., 1996). Arabidopsis ecotype Columbia-0 (Col-0) was used as the wild type in all experiments.

Plants were grown in soil or in hydroponic culture as previously described (Kölling et al., 2015) in a controlled environment chamber (KKD Hiross, CLITEC Boulaguiem) in a 12-h-light/12-h-dark cycle with a constant temperature of 23°C, 45% relative humidity, and a uniform illumination of 120 μmol m^{-2} s^{-1} (Osram L36W/965 Biolux and Osram L36/W77 Biolux at 3:1 ratio; continuous spectra). To induce osmotic stress, 3-week-old hydroponically grown plants were transferred to a nutrient solution supplemented with 300 mM mannitol or 300 mM sorbitol for 4 h, starting after 3 h of light. The rosettes and the roots were harvested separately at the indicated time points for further analyses. As a control, a subset of plants were kept in a nutrient solution without osmotic agents and harvested at the same time points. Alternatively, sterilized seeds were germinated on half-strength MS vertical plates, containing 0.8% (w/v) agar. Six days after germination, seedlings with the same root length were transferred for additional 9 d to MS plates optionally supplemented with 300 mM mannitol. Primary roots were measured using the ImageJ plug-in NeuronJ (Meijering et al., 2004) to determine growth rates. For ABA treatment, 3-week-old hydroponically grown plants were sprayed with 100 μM ABA-KOH in 0.01% (v/v) Tween 20, or a mock solution, starting after 3 h of light, and rosettes were harvested at different time points for further analyses.

qPCR Analysis of Transcript Levels

Total RNA was extracted from leaves using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Following DNase-I treatment, 1 μg of total RNA of each sample was used to produce cDNA using the M-MLV reverse transcriptase and oligo(dT) primers (Promega). Quantitative PCR was performed using SYBR green master mix with the 7500 Fast Real-Time PCR System (Applied Biosystems). Reactions were run in triplicate with three different cDNA preparations, and the instrument's iQ5 Optical System Software was used to determine the threshold cycle (Ct). Gene-specific transcripts were normalized to the *Actin2* gene (*ACT2*; At3g18780) and quantified by the ΔCt method (Ct of gene of interest – Ct of *ACT2* gene). Real-time SYBR green dissociation curves showed one species of amplicon for each primer combination listed in Supplemental Table 4.

Iodine Staining

Four-week-old Arabidopsis rosettes were harvested at the end of day or end of night and incubated in 80% (v/v) ethanol for 12 h to remove the chlorophyll. The cleared plants were rinsed in water and stained in Lugol's solution (Sigma-Aldrich) for 10 min.

Quantification of Starch and Leaf Soluble Sugars

Rosettes from plants grown in hydroponic culture or in soil were harvested into liquid N_2 and extracted in 0.7 M perchloric acid as previously described (Hostettler et al., 2011). Starch in the insoluble fraction was determined by measuring the amount of glucose released by treatment with α-amylase and amyloglucosidase (both from Roche). Sugars (maltose, glucose, fructose, and sucrose) in the soluble fraction were determined using HPAEC-PAD (Dionex ICS-5000; Thermo Scientific). Samples of the neutralized soluble fraction (200 μL) were applied to sequential 1.5-mL columns of cation exchanger Dowex 50 W and anion exchanger Dowex 1 (Sigma-Aldrich). Neutral compounds were eluted with 5 mL of water, lyophilized, redissolved in 200 μL of water, and separated on a CarboPac PA20 column on an ICS-3000 system (Dionex) as previously described (Egli et al., 2010). Peaks were identified by coelution with known malto-oligosaccharide standards and areas were determined using the instrument's Chromeleon software.

Quantification of Root Soluble Sugars

Root sugar measurements were performed on 3-week-old hydroponically grown plants optionally supplemented with 300 mM mannitol for 2 or 4 h. At the indicated time points, roots from three plants were harvested and pooled, rinsed briefly with deionized water to remove residual mannitol, weighed, and snap frozen in liquid N_2. Roots were pulverized while still frozen using the Mix Mill MM-301 (Retsch) and extracted in 1 mL 80% (v/v) ethanol for 15 min at 80°C. The pellet was sequentially washed with 0.5 mL 50% (v/v) ethanol, 20% (v/v) ethanol, and deionized water. The supernatants from each wash were pooled, dried under vacuum, and resuspended in 200 μL of water. Glucose, fructose, and sucrose were quantified enzymatically by adapting an existing method (Viola and Davies, 1992). Briefly, 15 μL of samples were added to 183 mL of 50 mM HEPES buffer, pH 7.5, containing 1 mM ATP, 1 mM NAD, and 1 mM $MgCl_2$. To measure glucose, hexokinase (Roche) and glucose 6-phosphate dehydrogenase (Roche) were used to convert glucose to 6-phosphogluconate with concomitant reduction of NAD to NADH, which was monitored spectrophotometrically at 340 nm. Subsequently, phosphoglucoisomerase (Roche) was added to determine the amount of fructose. Finally, invertase (Sigma-Aldrich) was added to cleave sucrose into fructose and glucose. The further increase in OD_{340} represented sucrose.

$^{14}CO_2$ Pulse-Chase Labeling

Whole-plant labeling experiments were performed with 3-week-old hydroponically grown plants optionally supplemented with 300 mM mannitol for 2 or 4 h. Plants were labeled as previously described (Kölling et al., 2015) using a sealed Plexiglas chamber illuminated with 120 μmol m^{-2} s^{-1}. $^{14}CO_2$ (300 μCi) was supplied for 60 min, either at the beginning of the stress (after 3 h of light) or in the middle of the stress treatment (after 5 h of light), after which the Plexiglas chamber was opened and the plants were kept in normal air for a chase period of 60 min (see Figure 3A for a schematic representation of the labeling set up). After the chase, the rosettes and the roots were harvested separately, the different tissues was fractionated between water soluble (neutral, acidic, and basic), ethanol soluble, and insoluble (starch and cell wall) compounds and the ^{14}C determined as described previously (Kölling et al., 2013).

Proline Quantification

Free proline content of rosettes and roots of hydroponically grown plants under control conditions or subject to mannitol stress was measured by adaptation of an existing method (Bates et al., 1973). In short, 200 μL of the soluble fraction from the sugar measurements were mixed with 800 μL of water, 1 mL of glacial acetic acid, and 1 mL of ninhydrin reagent (2.5% ninhydrin in a 6:3:1 mixture of glacial acetic acid:water:orthophosphoric acid). Samples were incubated for 1 h at 90°C, cooled to 25°C, combined with an equal volume of toluene, and mixed vigorously. Following phase partitioning, 1 mL of the upper organic phase was transferred into a quartz cuvette and the OD at 546 nm was measured spectrophotometrically. A calibration curve was prepared using different proline concentrations as a standard.

Measurements of Endogenous ABA Levels

Endogenous ABA content was measured in leaves of hydroponically grown plants with or without mannitol treatment for 4 h. Samples were extracted and measured as described in (Großkinsky et al., 2014).

Relative Water Content

Relative water content was measured in leaves of 3-week-old hydroponically grown plants with or without mannitol treatment and used as a measure of water loss in response to the stress. Rosettes were weighed at the time of sampling (fresh weight) and after incubation in deionized water for 24 h (rehydrated weight). Dry weight after 24 h at 50°C was also measured. Plant water status was evaluated from the relative water content [(fresh weight − dry weight)/(rehydrated weight − dry weight) * 100].

Osmolality (π)

For osmolyte concentration measurements, leaves from three plants were pooled, subject to five cycles of freezing/thawing, and subsequently mechanically ground. After centrifugation for 10 min at 16,000*g* at 4°C, 10 μL of the diluted supernatant (generally 1:3) was used to determine the osmolality using a Micro-Osmometer (Advance Instruments).

Determination of F_v/F_m and ΦPSII

Chlorophyll fluorescence transients of Col-0 and *amy3 bam1* leaves in response to osmotic stress were measured using the FluorCam 800MF (Photo Systems Instruments). The fluorescence measurement protocol uses short (30 μs) measuring flashes to measure the initial minimal fluorescence (F_o) emitted from dark-adapted leaves, followed by a strong saturating flash for 0.8 s measure the maximal fluorescence (F_m). After 15 min of dark adaptation, the leaf was exposed to actinic light for 4 min. Three strong flashes of saturating light probed the effective quantum yield (ΦPSII) during the actinic light exposure. The chlorophyll fluorescence transients were measured at the indicated time points in plants subject to 300 mM mannitol stress or kept in control nutrient solution. Image processing software integrated with the FluorCam (www.psi.cz) was used to process the captured time-resolved ChlF images. The numeric value of each ChlF parameter was determined by integrating it over the measured leaf area using the following formula: $F_v/F_m = (F_m - F_o)/F_m$; $\Phi PSII = F_m' - F'/F_m'$, where F_m' is the maximal fluorescence from light-adapted leaves and F' is the fluorescence emission from light-adapted leaves.

Stomatal Width

For stomatal width measurement, epidermal peels isolated from the abaxial side of the middle part of leaf 6 were glued onto a coverslip using a nontoxic medical adhesive (Medical Adhesive B Liquid, VM 355-1, Ulrich Swiss). The adaxial epidermis and mesophyll layers were gently removed. The coverslips with the glued abaxial epidermis were rinsed with 10 mM MES-KOH, pH 6.15, and stomata were immediately imaged using an inverted microscope (Nikon Eclipse TS100) at 40× magnification. Stomatal width was measured manually using ImageJ software (v 1.42q, NIH USA; http://

rsbweb.nih.gov/ij/). Around 20 pictures per leaf and per time point were taken, and more than 50 stomatal widths were measured.

Native PAGE and Activity Staining

Entire rosettes of hydroponically grown culture were harvested at the indicated time points and frozen in liquid N_2. Rosettes were ground using the Mix Mill MM-301 (Retsch) and incubated for 30 min at 4°C in extraction buffer (50 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM DTT, and 1× Complete EDTA Free Protease Inhibitor [Roche]; 300 μL per 100 mg fresh weight) for 30 min at 4°C. Insoluble material was removed by centrifugation at 15,000*g* for 5 min. Protein content was quantified using the BCA Protein Assay kit (Thermo Scientific), and 5 μg of protein was loaded onto the PAGE gels. For native PAGE, resolving gels contained 7.5% (w/v) acrylamide, 9% (v/v) glycerol, 375 mM Tris-HCl, pH 8.8, and 0.1% (w/v) amylopectin. The stacking gel contained 3.75% (w/v) acrylamide, 63 mM Tris-HCl, pH 6.8, and 0.1% (w/v) amylopectin. Protein extracts from leaves were mixed with PAGE loading buffer (final concentration 50 mM Tris-HCl, pH 6.8, 3% [v/v] glycerol, and 0.005% [w/v] bromophenol blue) and loaded onto the gel (5 μg protein). After 3 h electrophoresis at 4°C with a constant 120 V, the gels were washed once in a reducing incubation buffer (100 mM HEPES-KOH, pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM DTT) and then incubated for 2 h in the same buffer at 25°C. The gels were stained for 16 h at 4°C in Lugol's solution. Excess stain was removed by several washes in cold water.

Immunodetection of BAM1 and AMY3 Protein and Quantification

Rosettes of control and ABA-treated plants were harvested into microcentrifuge tubes containing three glass beads and kept frozen at −80°C prior to analysis. The tissue was ground into a fine powder using a Mix Mill. Protein extraction medium (40 mM Tris-HCl, pH 6.8, 5 mM $MgCl_2$, and Protease inhibitor cocktail [Roche]) was added to the powder at a ratio of 1 mL per 100 mg tissue. Insoluble material was spun down at 20,000*g* for 5 min, and soluble proteins were collected in the supernatant. Protein (5 μg) was loaded onto SDS-PAGE gels and electrophoresis performed using standard protocols. For immunoblotting, proteins were transferred onto a PVDF membrane following SDS-PAGE. The BAM1 protein was detected using a polyclonal rabbit antibody (Eurogentec) raised against a recombinant His-tagged Arabidopsis BAM1 protein, which was expressed and purified from *Escherichia coli*. Antibodies specific for BAM1 were affinity purified from the antiserum against the recombinant BAM1 protein conjugated to NHS-activated Sepharose (GE healthcare). The purified antibody was used at a dilution of 1:10,000. AMY3 was detected using polyclonal antibodies raised against recombinant AMY3 protein (Yu et al., 2005), at a dilution of 1:3000. Bands were visualized by chemiluminescence detection. Quantification of band intensities was conducted using the densitometry feature of ImageJ software.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL libraries under accession numbers: *ACT2* (At3g18780), *RD29A*, (At5g52310), *BAM1* (At3g23920), *AMY3* (At1g69830), *BAM3* (At4g17090), *PGM* (At5g51820), *NCED3* (At3g14440), *ABA2* (At1g52340), *AAO3* (At2g27150), *AREB1* (At1g45249), *AREB2* (At3g19290), *ABF1* (At1g49720), *ABF3* (At4g34000), *SNRK2.2* (At3g50500), *SNRK2.3* (At5g66880), and *SNRK2.6* (At4g33950).

Supplemental Data

Supplemental Figure 1. Starch and maltose levels in wild-type plants upon mannitol or sorbitol treatment.

Supplemental Figure 2. Starch and maltose levels in wild-type and *pgm* mutant plants upon mannitol treatment.

Supplemental Figure 3. Impact of simultaneous loss of BAM1 and AMY3 on starch metabolism in plants grown under control conditions.

Supplemental Figure 4. Expression levels of *BAM1* in wild-type and *bam3* mutant plants.

Supplemental Figure 5. AMY3 is a highly abundant protein in plant leaves.

Supplemental Figure 6. Water absorption by wild-type and *amy3 bam1* plants in response to mannitol stress.

Supplemental Figure 7. Soluble sugars and proline content of shoots in response to osmotic stress.

Supplemental Figure 8. Accumulation of endogenous ABA in response to mannitol treatment.

Supplemental Figure 9. Quantification of BAM1 protein in leaves of wild-type plants treated with ABA.

Supplemental Figure 10. Starch and maltose levels in wild-type and *aba2* mutant plants upon mannitol treatment.

Supplemental Figure 11. Starch levels in response to mannitol, ABA, or a combination of mannitol and ABA treatment.

Supplemental Figure 12. Expression levels of *RD29A*, *BAM1*, *BAM3*, and *AMY3* in *snrk2.2 snrk2.3 snrk2.6* and *areb1 areb2 abf3* mutants under control conditions.

Supplemental Figure 13. *BAM1* and *BAM3* are differentially regulated by abiotic stresses.

Supplemental Table 1. Carbon partitioning into the major cellular compound classes in shoots and roots of plants labeled at the beginning of the stress treatment.

Supplemental Table 2. Carbon partitioning into the major cellular compound classes in shoots and roots of plants labeled in the middle of the stress treatment.

Supplemental Table 3. Chlorophyll *a* fluorescence parameters of wild-type and *amy3 bam1* plants subject to osmotic stress.

Supplemental Table 4. Sequences of primers used in this work.

Supplemental Data Set 1. Promoter sequences used for the MEME analysis in Figure 8.

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AUTHOR CONTRIBUTIONS

D. Santelia conceived the project. D. Santelia and M.T. designed the experiments. D. Santelia, M.T., D.P., D. Seung, D.H., A.N., T.M., K.K.,

and H.W.P. performed the experiments and analyzed the data. D. Santelia, M.T., and S.C.Z. wrote the article.

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Regulation of Leaf Starch Degradation by Abscisic Acid Is Important for Osmotic Stress Tolerance in Plants

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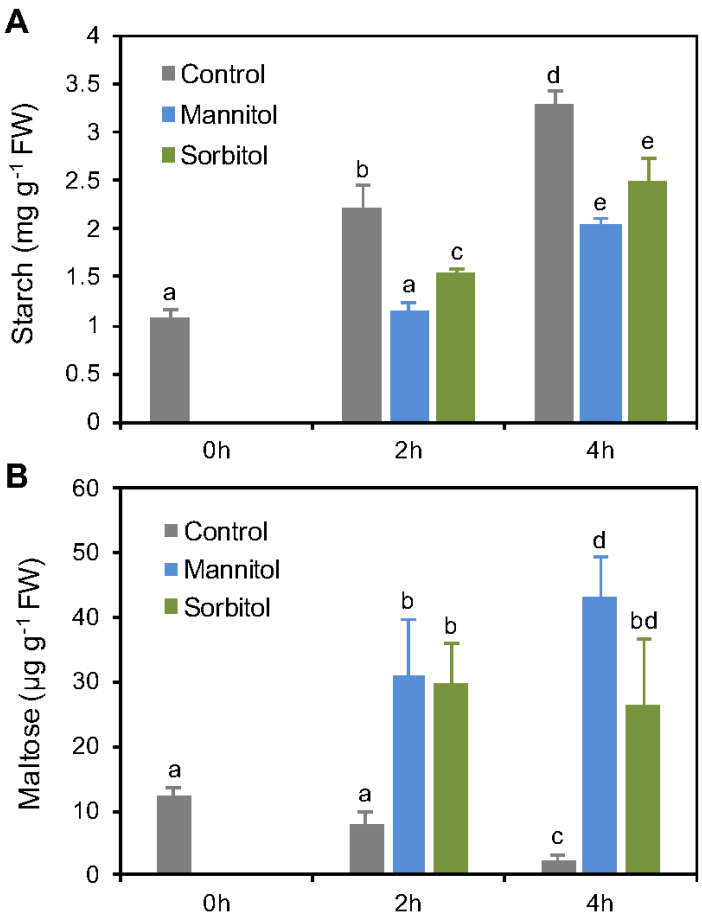

Supplemental Figure 1. Starch and maltose levels in WT plants upon mannitol or sorbitol treatment.
Leaf starch **(A)** and maltose **(B)** content in WT Arabidopsis plants treated with 300 mM mannitol or 300 mM sorbitol for 4 h compared with controls. Each value is the mean ± SE (n = 6). FW, fresh weight. Statistical significances determined by unpaired two-tailed Student's t tests: different letters denote $p < 0.05$.

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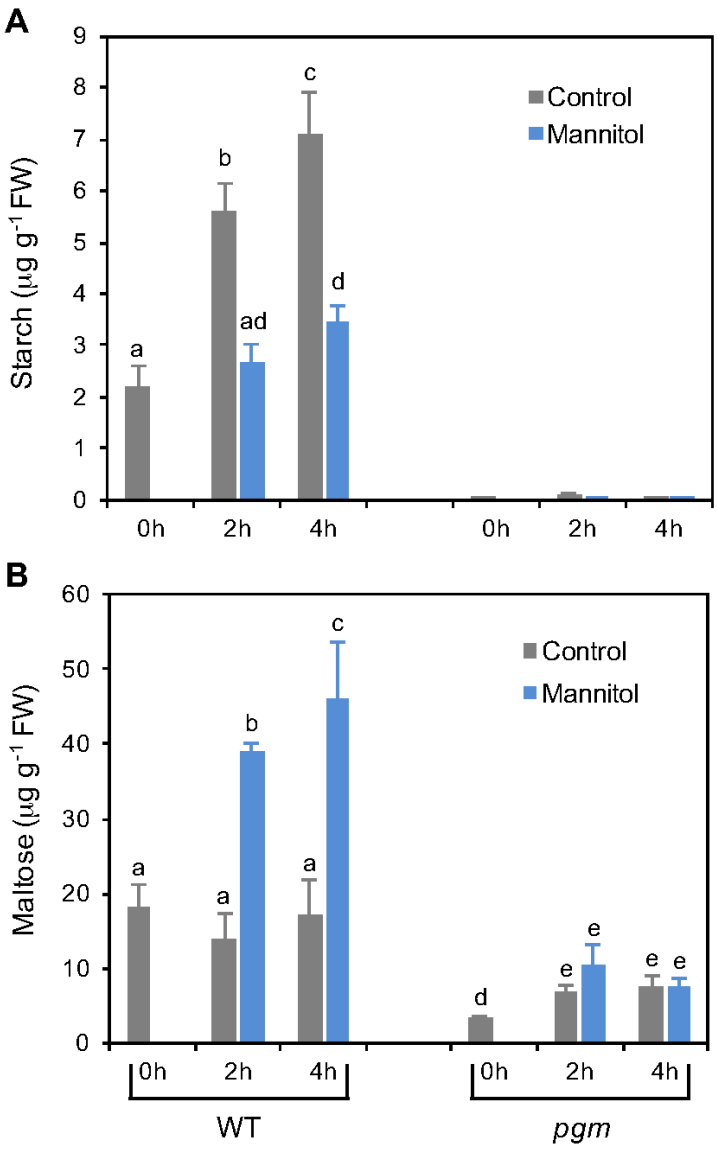

Supplemental Figure 2. Starch and maltose levels in WT and *pgm* mutant plants upon mannitol treatment.
Leaf starch **(A)** and maltose **(B)** content in WT and *pgm* Arabidopsis mutant plants treated with 300 mM mannitol for 4 h compared with controls. Each value is the mean ± SE (n = 6). FW, fresh weight. Statistical significances determined by unpaired two-tailed Student's t tests: different letters denote $p < 0.05$.

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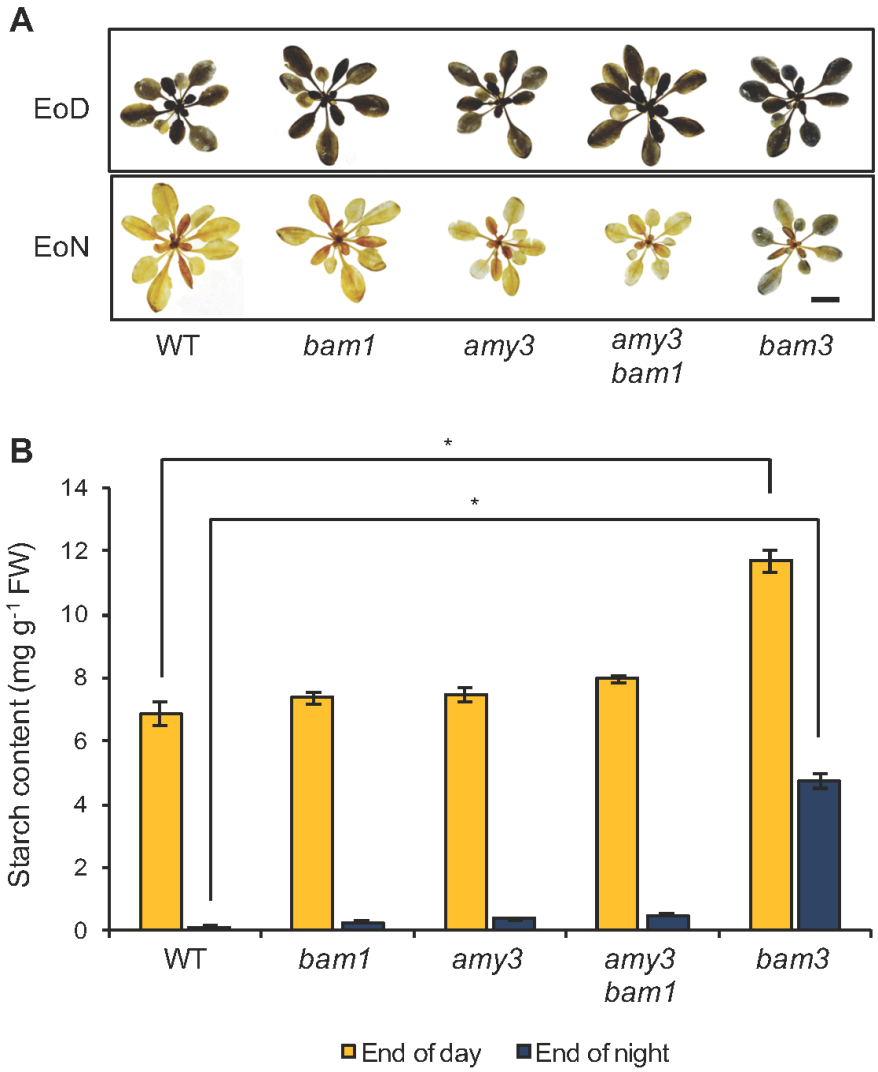

Supplemental Figure 3. Impact of simultaneous loss of BAM1 and AMY3 on starch metabolism in plants grown under control conditions.

(A) Photographs of iodine-stained WT, *bam1*, *amy3*, *amy3 bam1* and *bam3* rosettes harvested at the end of the day (EoD) and at the end of the night (EoN) after 4 weeks of growth. Bar = 1 cm.

(B) Starch content at the end of the day and at the end of the night in WT, *bam1*, *amy3*, *amy3 bam1* and *bam3* leaves. Values are means ± SE (n = 8). FW, fresh weight. Statistical significances determined by unpaired two-tailed Student's t tests: * denotes $p < 0.05$ for the indicated comparison.

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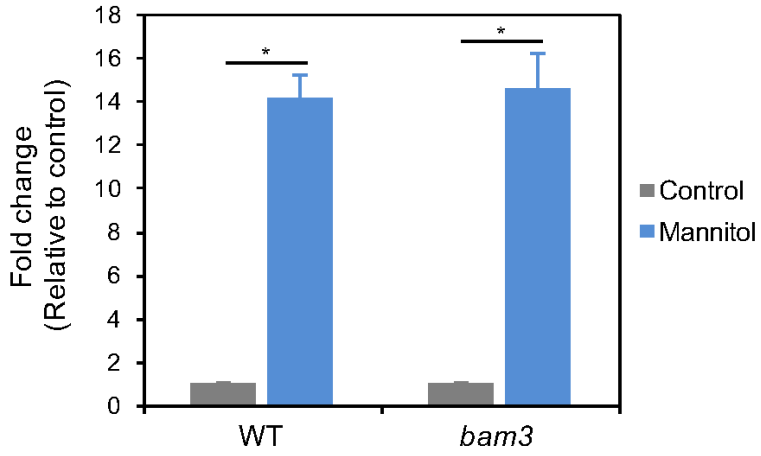

Supplemental Figure 4. Expression levels of *BAM1* in WT and *bam3* mutant plants.
Hydroponically-grown WT and *bam3* plants were transferred to fresh nutrient solution (control) or nutrient solution containing 300 mM mannitol for 4 h. *BAM1* transcript abundance, determined by qPCR, was compared in osmotically-stressed and control leaves. The *ACT2* gene served as a reference gene. Values representing means ± SE (n = 3) were normalized against gene expression in control conditions (set as 1). Statistical significances determined by unpaired two-tailed Student's t tests: * denotes $p < 0.05$ for the indicated comparison.

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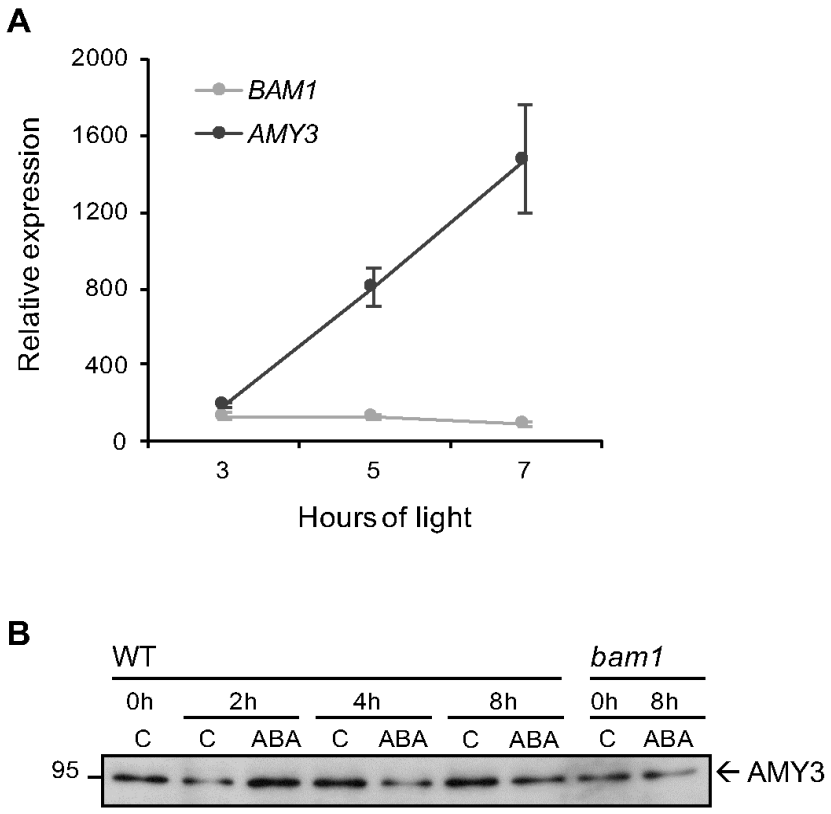

Supplemental Figure 5. AMY3 is a highly abundant protein in plant leaves.
(A) Relative *AMY3* and *BAM1* transcript abundance was determined in hydroponically-grown WT after 3 h, 5 h and 7 h of light, using qPCR. The *ACT2* gene served as a reference gene. Values are means ± SE (n = 3).
(B) Immunodetection of AMY3 protein in leaves of WT and *bam1* plants treated with ABA. Total protein was extracted from entire rosettes of hydroponically-grown plants at the indicated time points after treatment with 100 μM ABA. Equal amounts of protein (5 μg) were separated by SDS-PAGE. AMY3 was detected by immunoblotting using antibodies raised against the recombinant AMY3 protein. No differences in AMY3 protein abundance were observed between control (C) and ABA-treated plants. Replicate blots yielded the same result. The loading control for this blot is the same as the one presented in Figure 4B, as the two experiments were performed in parallel.

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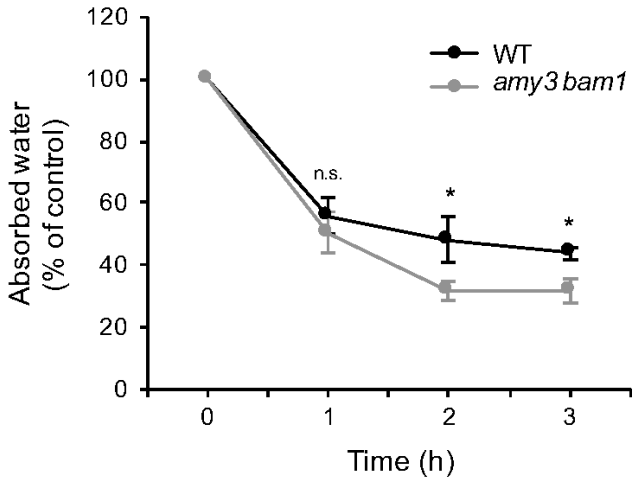

Supplemental Figure 6. Water absorption by WT and *amy3 bam1* plants in response to mannitol stress.
The volume of water absorbed by plants in a nutrient solution supplemented with 300 mM mannitol is shown as a percentage of water absorbed by plants in a solution without mannitol (control, set as 100%). Values were corrected for water loss due to the evaporation from the liquid surface. Values are means ± SE (n = 6). Statistical significances determined by unpaired two-tailed Student's t tests: * denotes $p < 0.05$ for the indicated comparison; n.s. = not significant for the indicated comparison.

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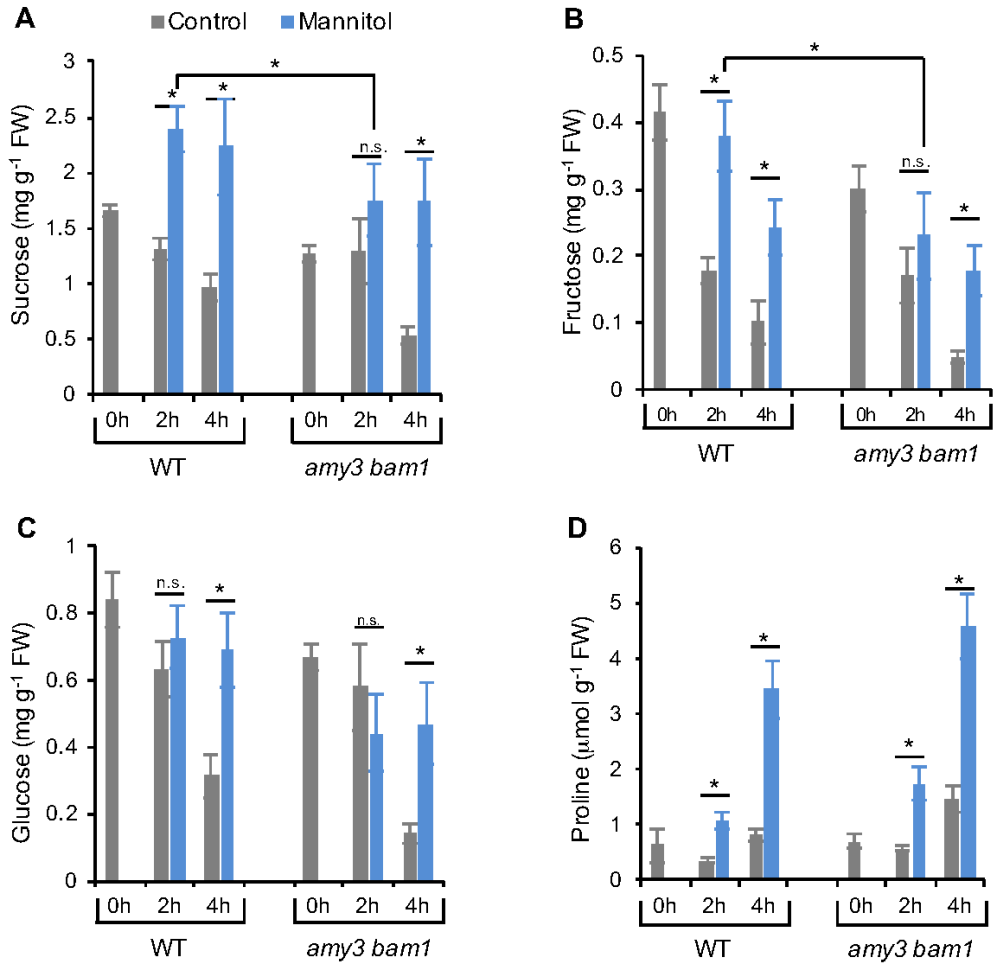

Supplemental Figure 7. Soluble sugars and proline content of shoots in response to osmotic stress.
Sucrose **(A)**, fructose **(B)**, glucose **(C)** and proline **(D)** content in the shoots of WT and *amy3 bam1* plants in response to osmotic stress treatment. Hydroponically-grown plants were optionally supplemented with 300 mM mannitol for 4 h. Sugars were measured at the indicated time points using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Proline was measured as described in Methods. Values are means ± SE (n = 6). FW, fresh weight. Statistical significances determined by unpaired two-tailed Student's t tests: * denotes $p < 0.05$ for the indicated comparison; n.s. = not significant for the indicated comparison.

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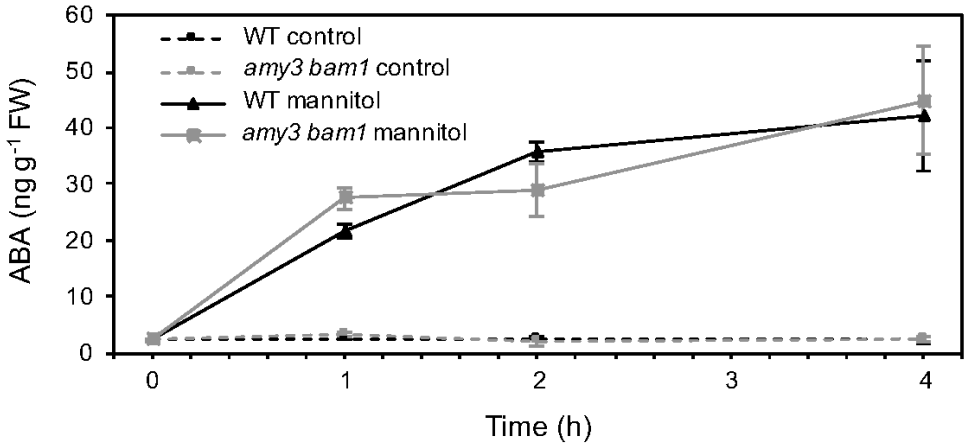
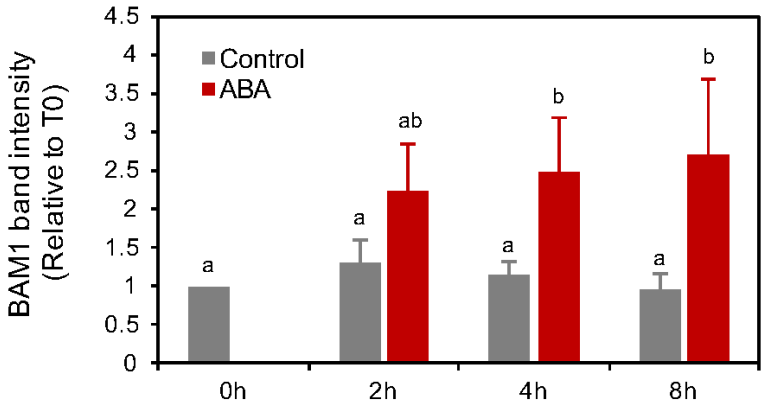


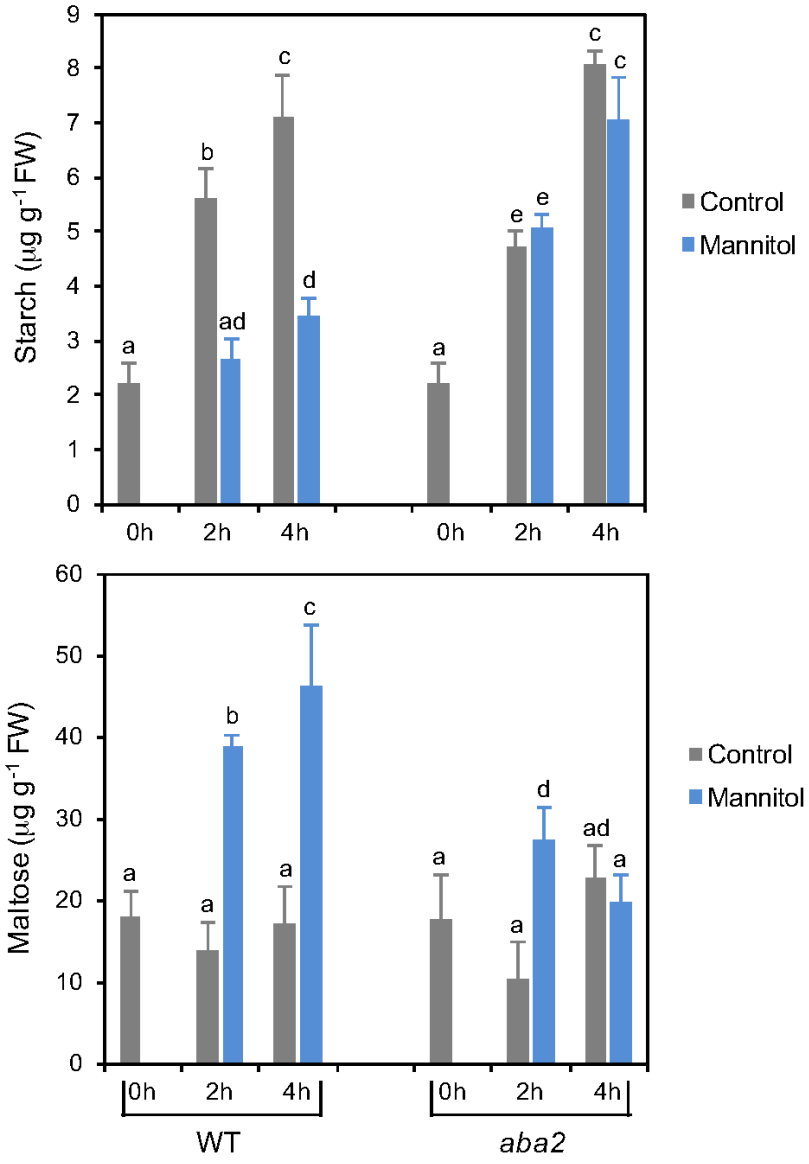
Supplemental Figure 8. Accumulation of endogenous ABA in response to mannitol treatment.
ABA levels in rosette of hydroponically-grown WT and *amy3 bam1* plants subject to 300 mM mannitol stress for 4 h were determined at the indicated time points as described in Methods. Values are means ± SE (*n* = 4). FW, fresh weight.

Supplemental Data. Thalmann et al. Plant Cell (2016) 10.1105/tpc.16.00143



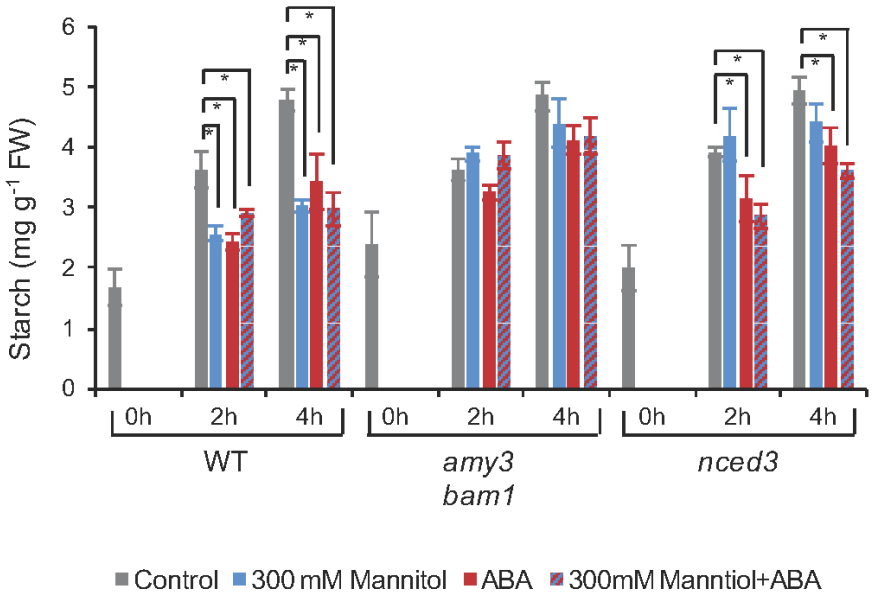
Supplemental figure 9. Quantification of BAM1 protein in leaves of WT plants treated with ABA.
Densitometry analysis (ImageJ) of three replicate blots was used to quantify band intensities. Values are expressed relative to the mean band intensity at time 0 (T0, set as 1). Each value is the mean ± SE of three biological samples. Each sample was blotted in three technical replicates. Statistical significances determined by unpaired two-tailed Student's *t* tests: different letters denote $p < 0.05$.

Supplemental Data. Thalmann et al. Plant Cell (2016) 10.1105/tpc.16.00143



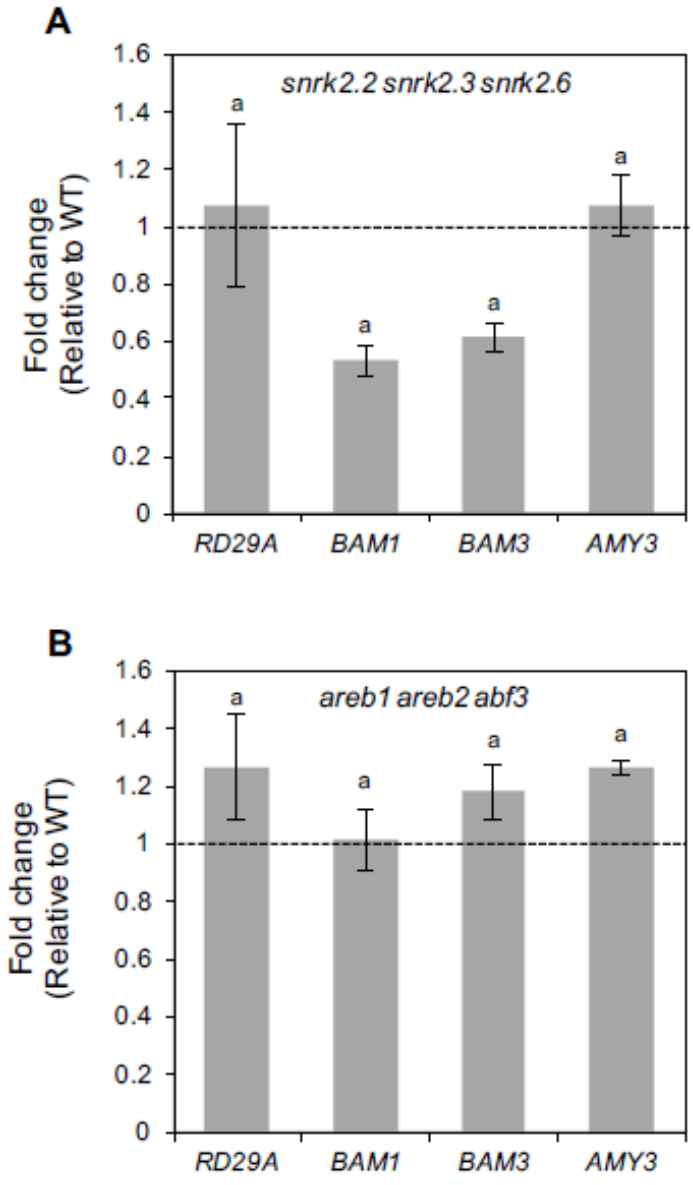
Supplemental Figure 10. Starch and maltose levels in WT and *aba2* mutant plants upon mannitol treatment.
Leaf starch **(A)** and maltose **(B)** content in WT and *aba2* Arabidopsis mutant plants treated with 300 mM mannitol for 4 h compared with controls. Each value is the mean ± SE (n = 6). FW, fresh weight. Starch and maltose content in WT is the same as in Supplemental Figure 2, as the experiments were conducted in parallel. Statistical significances determined by unpaired two-tailed Student's t tests: different letters denote $p < 0.05$

Supplemental Data. Thalmann et al. Plant Cell (2016) 10.1105/tpc.16.00143



Supplemental Figure 11. Starch levels in response to mannitol, ABA or a combination of mannitol and ABA treatment.
Leaf starch in WT, *amy3 bam1* and *nced3* plants treated with 300 mM mannitol, 100 μM ABA or 300 mM mannitol + 100 μM ABA for 4 h, compared with controls. Values are means ± SE (n = 6). FW, fresh weight. Statistical significances determined by unpaired two-tailed Student's *t* tests: * denotes $p < 0.05$ for the indicated comparison.

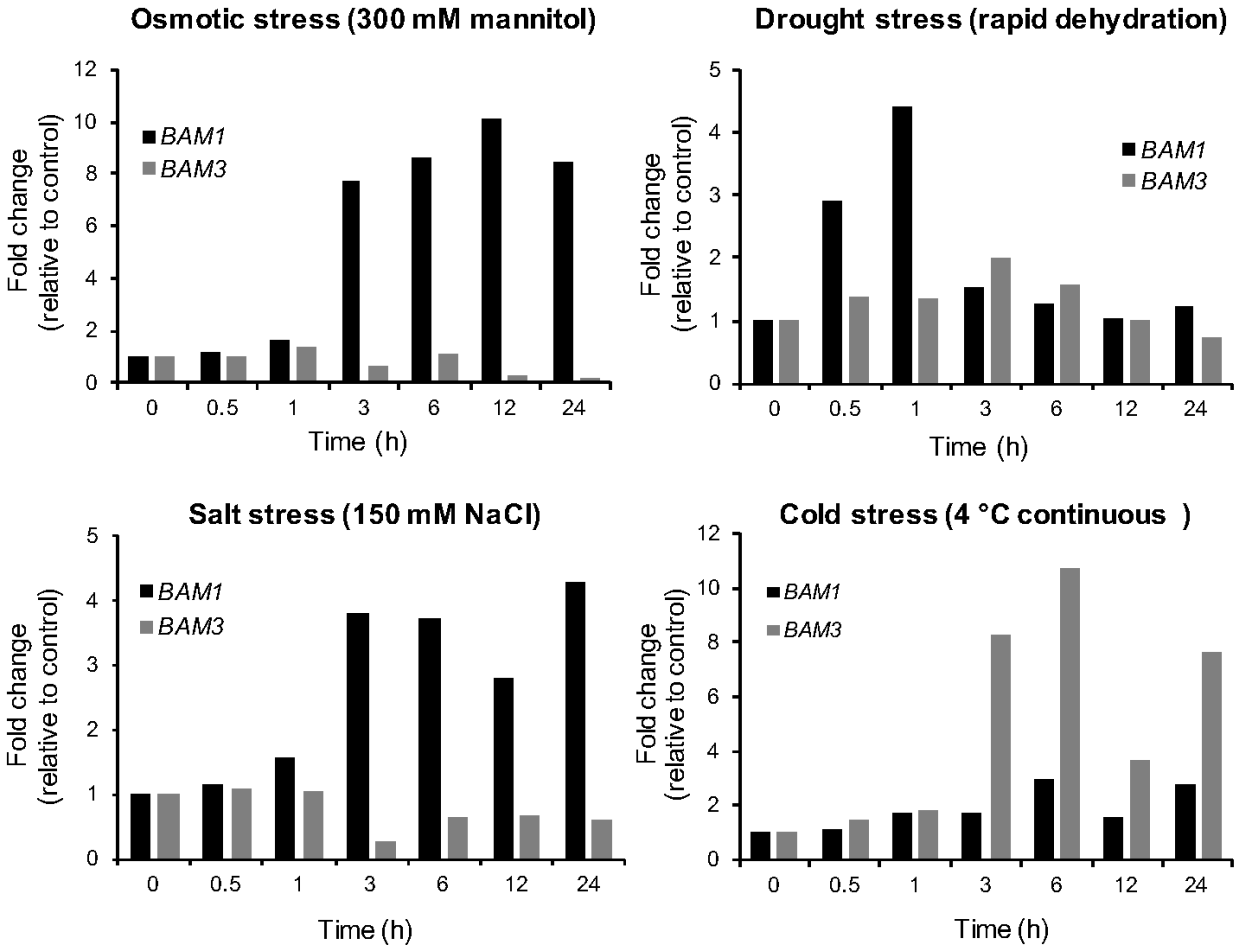
Supplemental Data. Thalmann et al. Plant Cell (2016) 10.1105/tpc.16.00143



Supplemental Figure 12. Expression levels of *RD29A*, *BAM1*, *BAM3* and *AMY3* in *snrk2.2 snrk2.3 snrk2.6* and *areb1 areb2 abf3* mutants under control conditions.

Leaf transcript abundance was determined in hydroponically-grown WT and *snrk2.2 snrk2.3 snrk2.6* **(A)** or *areb1 areb2 abf3* **(B)** mutants after 3 h of light, using qPCR. The *ACT2* gene was used as reference gene. Expression values are given relative to the levels in WT (set as 1). Values are means ± SE (n = 3). No statistically significant differences were observed, as determined by unpaired two-tailed Student's *t* tests.

Supplemental Data. Thalmann et al. Plant Cell (2016) 10.1105/tpc.16.00143



Supplemental Figure 13. *BAM1* and *BAM3* are differentially regulated by abiotic stresses.
Comparison of expression profiles of *BAM1* and *BAM3* in response to various abiotic stresses (drought, salt, cold and osmotic stress). Data were retrieved from the public eFP browser microarray dataset 'Abiotic Stress' (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

Supplemental Data. Thalmann et al. Plant Cell (2016) 10.1105/tpc.16.00143

% of ^{14}C recovered in each fraction related to the total amount per tissue (2 h stress)

	WT		*amy3 bam1*	
	Control	**Mannitol**	**Control**	**Mannitol**
Root	8.21±0.65	25.88±3.86	7.76±0.63	23.20±0.28
Water soluble	5.00±0.40	23.62±3.71	4.25±0.31	20.48±0.30
Acidic	0.33±0.02	0.71±0.33	0.89±0.06	1.51±0.04
Basic	1.68±0.13	2.30±0.22	1.37±0.11	2.24±0.08
Neutral	2.96±0.28	20.50±3.28	1.96±0.16	16.64±0.29
Nucleotide	0.035±0.002	0.097±0.032	0.027±0.002	0.083±0.03
Water insoluble	3.21±0.33	2.26±0.34	3.51±0.32	2.73±0.26
Starch	-0.11±0.11	0.02±0.04	-0.06±0.04	0.04±0.07
Protein	0.87±0.12	0.41±0.19	1.06±0.34	1.01±0.47
Remaining	2.45±0.37	1.83±0.39	2.51±0.47	1.67±0.54
Shoot	91.79±3.69	74.12±4.13	92.24±1.65	76.80±1.28
Ethanol soluble	10.57±0.75	8.83±0.73	10.34±0.37	8.31±0.06
Water soluble	29.80±3.08	26.74±2.61	29.71±1.04	31.62±0.82
Acidic	8.43±0.77	6.47±1.58	8.41±0.39	6.01±0.45
Basic	13.72±1.62	9.72±1.35	13.19±0.40	10.96±0.57
Neutral	6.78±0.69	9.76±0.92	7.19±0.45	13.82±0.71
Nucleotide	0.87±0.08	0.79±0.11	0.92±0.06	0.82±0.03
Water insoluble	51.42±1.89	38.55±3.12	52.19±1.23	36.87±0.98
Starch	32.41±1.00	21.70±3.54	31.99±1.69	22.01±1.01
Protein	7.15±0.28	5.74±0.51	8.09±0.26	6.40±0.13
Remaining	11.86±1.11	11.10±0.67	12.11±0.62	8.46±0.38

Supplemental Table 1. Carbon partitioning into the major cellular compound classes in shoot and root of plants labelled at the beginning of the stress treatment.

Whole WT, and *amy3 bam1* plants were labelled with $^{14}CO_2$ for 1 h, just after transfer to a mannitol containing nutrient solution. Following a 1-h chase period, the shoot and root were harvested separately and the amount of ^{14}C incorporated into the different compound classes in each organ was determined by liquid scintillation counting. The sum of the label in each organ is set to 100%. The relative amount of ^{14}C incorporated into the different compound classes (as a percentage of the total label in the corresponding organ) is shown. Values are means ± SE (n = 4).

Supplemental Data. Thalmann et al. Plant Cell (2016) 10.1105/tpc.16.00143

% of ^{14}C recovered in each fraction related to the total amount per tissue (4 h stress)

	WT		*amy3 bam1*	
	Control	**Mannitol**	**Control**	**Mannitol**
Root	7.01±0.25	16.52±1.56	8.65±1.88	9.44±1.37
Water soluble	4.31±0.23	14.86±1.41	4.21±0.67	8.09±1.25
Acidic	1.21±0.05	1.00±0.10	1.43±0.21	0.88±0.15
Basic	0.94±0.12	1.01±0.14	1.10±0.20	1.03±0.19
Neutral	2.13±0.14	12.83±1.24	1.65±0.26	6.17±0.94
Nucleotide	0.015±0.002	0.014±0.001	0.027±0.006	0.013±0.002
Water insoluble	2.71±0.09	1.66±0.17	3.58±0.47	1.34±0.17
Starch	0.06±0.06	0.09±0.04	0.00±0.01	0.03±0.02
Protein	0.60±0.05	0.30±0.07	0.61±0.09	0.36±0.05
Remaining	2.05±0.12	1.27±0.18	2.96±0.48	0.95±0.18
Shoot	92.99±2.80	83.48±2.01	91.35±2.65	90.56±2.86
Ethanol soluble	8.29±0.30	6.77±0.03	8.11±0.68	6.57±0.29
Water soluble	29.27±2.15	34.36±1.72	32.34±1.06	38.11±1.93
Acidic	14.85±1.46	11.33±0.63	14.74±0.50	15.07±0.96
Basic	6.29±0.45	5.24±0.24	8.54±0.45	6.86±0.31
Neutral	7.18±0.45	17.10±1.75	8.02±1.19	15.42±2.15
Nucleotide	0.95±0.06	0.69±0.03	1.04±0.02	0.76±0.04
Water insoluble	55.42±1.76	42.35±1.03	50.91±2.33	45.89±2.09
Starch	38.83±1.75	30.90±0.96	36.17±1.82	34.93±1.84
Protein	7.36±0.12	5.91±0.11	6.78±0.65	5.32±0.14
Remaining	9.24±0.41	5.54±0.25	7.96±1.47	5.64±2.77

Supplemental Table 2. Carbon partitioning into the major cellular compound classes in shoot and root of plants labelled in the middle of the stress treatment.

Whole WT and *amy3 bam1* plants were labelled with $^{14}CO_2$ for 1 h, in the middle of the stress treatment. Following a 1-h chase period, the shoot and root were harvested separately and the amount of ^{14}C incorporated into the different compound classes in each organ was determined by liquid scintillation counting. The sum of the label in each organ is set to 100%. The relative amount of ^{14}C incorporated into the different compound classes (as a percentage of the total label in the corresponding organ) is shown. Values are means ± SE (n = 4).

Supplemental Data. Thalmann et al. Plant Cell (2016) 10.1105/tpc.16.00143

Chlorophyll *a* fluorescence parameters of Col-0 and *amy3 bam1* plants subject to osmotic stress

		F_v/F_m		ΦPSII	
		Control	**Mannitol**	**Control**	**Mannitol**
WT	0 h	0.817±0.003		0.658±0.003	
	2 h	0.815±0.002	0.767±0.011*	0.647±0.002	0.602±0.010*
	4 h	0.817±0.002	0.802±0.002	0.648±0.002	0.635±0.004
	24 h	0.807±0.003	0.811±0.003	0.640±0.001	0.649±0.004
amy3 bam1	0 h	0.818±0.003		0.655±0.003	
	2 h	0.811±0.003	0.767±0.010*	0.644±0.003	0.602±0.011*
	4 h	0.813±0.003	0.802±0.004	0.648±0.005	0.637±0.005
	24 h	0.810±0.006	0.813±0.002	0.635±0.005	0.651±0.002

Supplemental Table 3. Chlorophyll *a* fluorescence parameters of Col-0 and *amy3 bam1* plants subject to osmotic stress.

The chlorophyll fluorescence transients F_v/F_m and ΦPSII were measured at the indicated time points in Col-0 and *amy3 bam1* plants subject to 300 mM mannitol stress or kept in control nutrient solution, using a FluorCam. The plants were dark adapted for about 15 min prior to each measurement. Values are means ± SE of four independent determinations. Asterisks indicate statistically significant differences between control and osmotic-stressed plants for each genotype, determined by Student's *t* tests (*P value < 0.05). No statistically significant differences were observed between Col-0 and *amy3 bam1* mutant plants.

The maximum quantum efficiency of PSII photochemistry (F_v/F_m) was reduced in both genotypes at a similar level after 2 h (0.767±0.010 and 0.767±0.011 for *amy3 bam1* and wild-type plants, respectively). A reduction was also observed in the PSII operating efficiency (ΦPSII), indicating a decrease in the relative quantum yield of linear electron transfer through the photosystems. This suggests that osmotic stress led to transient negative effects on PSII efficiency, but to a similar extent in the two genotypes.

Supplemental Data. Thalmann et al. Plant Cell (2016) 10.1105/tpc.16.00143

Supplemental Table 4. Sequences of primers used in this work.

Primer	Sequence
Primers used for genotyping T-DNA insertion lines	
***bam1* (Salk_039895)**	
BAM1 Salk_fw	CCATTGTGGAAATCCAAGTG
BAM1 Salk_rev	ACGAGTACTTATCATAGCACTG
Salk LBb1.3	ATTTTGCCGATTTCGGAAC
***amy3* (Sail_613 D12)**	
AMY3 Sail_fw	GGTTCCTCTTGTAGACGATGTTCC
AMY3 Sail_rev	CCGACCTTGTGAAATTTCTTCACTG
Sail LBb	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
***nced3* (GABI_129B08)**	
NCED3 GABI_fw	GTCAGCCACGAGAAGCTACAC
NCED3 GABI_rev	ACAGAGGCTCTCCTCCGTAAC
GABI LBb	ATATTGACCATCATACTCATTGC
Primers used for qPCR	
***BAM1* (AT3G23920)**	
BAM1 RT_fw	CCATTGTGGAAATCCAAGTG
BAM1 RT_rev	ACGAGTACTTATCATAGCACTG
***AMY3* (AT1G69830)**	
AMY3 RT_fw	TGCTTACATCCTAACTCATCC
AMY3 RT_rev	CTCTTGTCTATATTCACCTCACTC
***BAM3* (AT4G17090)**	
BAM3 RT_fw	TGATTCTGTGCCTGTCCT
BAM3 RT_rev	GAATTTCCGCAATAACTCCTC
***RD29A* (AT5G52310)**	
RD29A RT_fw	CCCACCAAAGAAGAAACTGGA
RD29A RT_rev	TTCAAATTGTCCTGGCTTCTG
***Actin2* (AT3G18780)**	
Actin RT_fw	TGGAATCCACGAGACAACCTA
Actin RT_rev	TTCTGTGAACGATTCCTGGAC

Addendum to chapter 1

In addition to the results published in Thalmann et al (2016), we conducted several experiments investigating whether other starch enzymes besides BAM1 and AMY3 were also involved in stress-induced starch degradation. As a comparison, we also imposed osmotic stress using different osmolytes. The results of these experiments are briefly summarized here.

Transcriptional analysis of selected starch-related genes in response to osmotic stress

To investigate if beside AMY3 and BAM1 additional enzymes are involved in stress-induced starch degradation, we analysed the induction of two debranching enzymes, *ISA3* and *LDA*, as well as of *PHS1* (Fig. 1). *ISA3* was strongly induced by osmotic stress, while *PHS1* and *LDA* were only mildly induced.

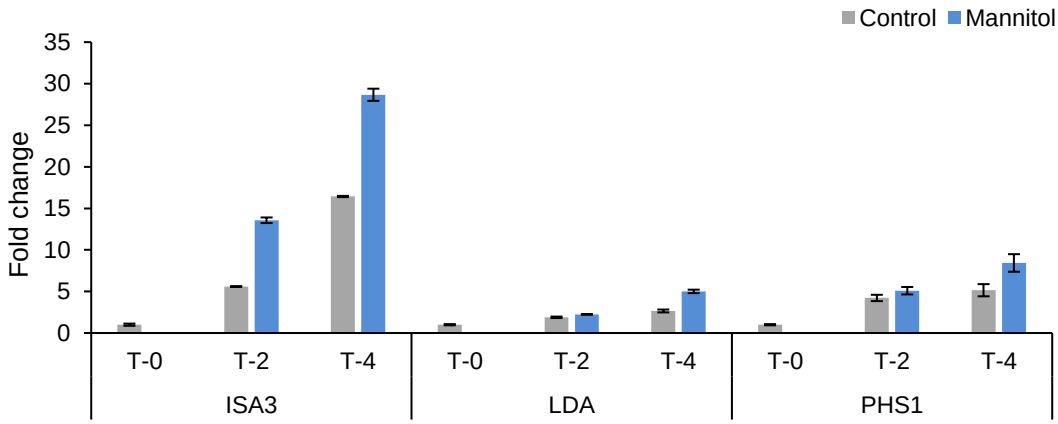


Figure 1: Leaf transcript abundance for *ISA1*, *LDA*, and *AMY3* in osmotically stressed and control leaves, determined by qPCR. Plants grown as in Thalmann et al. (2016) were harvested at the indicated time points. The *ACT2* gene was used as a reference gene.

This indicates that in addition to AMY3 and BAM1 other enzymes are involved in stress-induced starch degradation. The involvement of a debranching enzyme could already be inferred, as the AMY3 alone can release branched oligosaccharides from starch, as detected by HPLC analysis (Streb et al., 2012; Seung et al., 2013). However, in Thalmann et al. (2016) no such compounds were identified during maltose quantification from crude extracts of osmotic-stressed rosettes, suggesting that they had been degraded by debranching enzymes. Interestingly, ISA3 is not only induced by osmotic stress but also

essential for night-time starch turnover (Delatte et al., 2006). Thus, it appears that unlike the specialisation seen in β-amylases the same debranching enzyme is involved in both stress-induced and nocturnal starch degradation. A similar situation was observed in guard cells, where starch degradation depends on BAM1 but not BAM3, while ISA3 is still the major debranching enzyme (Horrer et al., 2016). It has previously been reported that the *phs1* mutant is more susceptible to water stress (Zeeman et al., 2004), and the observed induction of *PHS1* by osmotic stress provides further evidence for an important role of this enzyme during water stress. To further investigate the function of PSH1, the response of *phs1* as well as *amy3/bam1/phs1* mutants to osmotic stress should be investigated.

In addition to RD29A we also tested the expression of another known stress-induced gene, RAB18, in WT, *amy3/bam1* and *bam3* mutant plants (Fig 2). Both genes were induced in all lines, indicating that the mutants are not impaired in stress-induced transcriptional changes.

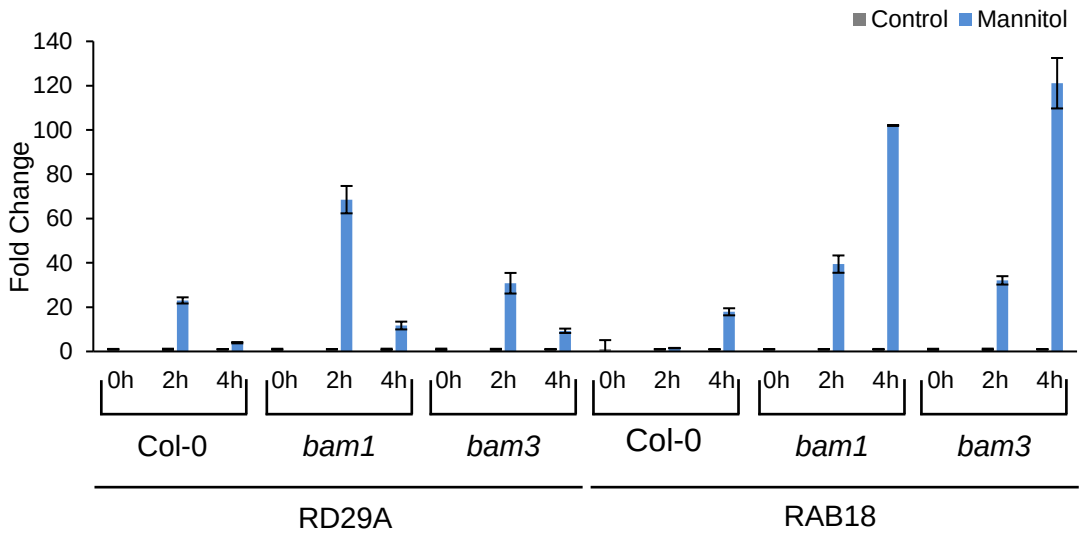


Figure 2: Leaf transcript abundance for *RD29A* and *RAB18* in osmotically stressed and control leaves of indicated genotypes, determined by qPCR. Plants grown as Thalmann et al. (2016) were harvested at the indicated time points. The *ACT2* gene was used as a reference gene.

Stress-induced starch degradation is blocked in bam1/bam3 *mutants*

It was previously described that the *bam1/bam3* double mutant shows a more severe starch excess than the *bam3* single mutant, although the *bam1* single mutant has no starch excess (Fulton et al., 2008). Thus it appears that BAM1 can partially compensate for the loss of BAM3 during nocturnal starch

degradation. We investigated whether a similar compensation would occur during stress-induced starch degradation, and subjected *bam1/bam3* mutants to osmotic stress as described in the publication. As expected, the starch content in the double mutant was much higher than in Col-0 and either single mutant (Fig 3). Interestingly, the residual starch degradation still observed in *bam1* mutants was no longer detectable in the double mutant. Thus, it appears that *bam3* can contribute to stress-induced starch degradation, in the absence of BAM1. This contrasts with the situation in stomata where the loss of BAM3 in the *bam1* background did not aggravate the starch excess phenotype (Horrer et al., 2016).

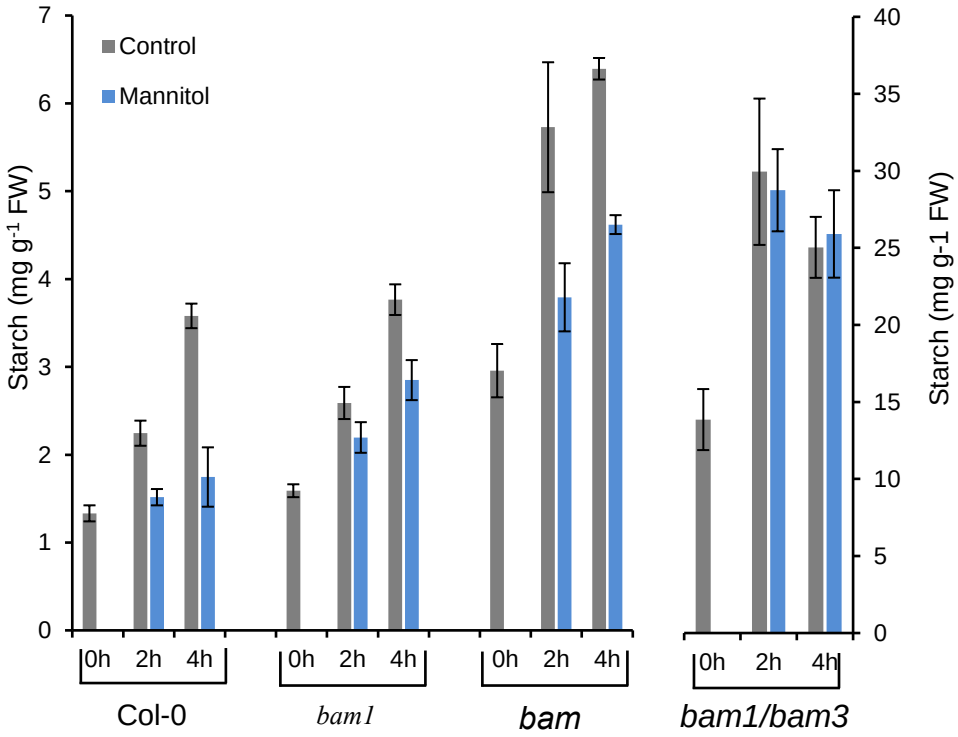


Figure 3: Leaf starch content of osmotically stressed leaves compared with controls. Values are means ± SE (n = 8). FW, fresh weight. Values for Col-0, *bam1*, and *bam3* are the same as in Fig. 1 B of (Thalmann et al.,

Changes of stress-induced starch metabolism in two starch synthesis mutants

Although we could show that starch is degraded during osmotic stress, the incorporation of ^{14}C into starch during stress as presented in supplementary table 1 and 2 in Thalmann et al (2016) indicates that starch synthesis continues nonetheless. We wanted to investigate if the starch synthesis during stress relies on the same pathway that operates under non-stress conditions. We subjected mutants deficient in different isoforms of the AGPase large subunit

(APL). In unstressed plants APL1 – also known as ADG2 – is the major isoform and mutants lacking this subunit accumulate less starch than wild type plants (Lin et al., 1988; Wang et al., 1997). In addition to APL1, three other isoforms exist (Crevillén et al., 2003). One of these, APL4, has been implicated in resistance to oxidative stress (Sulmon et al., 2011).

We first tested whether *APL1* and *APL4* expression was changed by osmotic stress using qPCR. Expression of *APL1* was unchanged by osmotic stress. In contrast expression of *APL4* was very low under control condition but increased strongly upon exposure to stress (Fig 4).

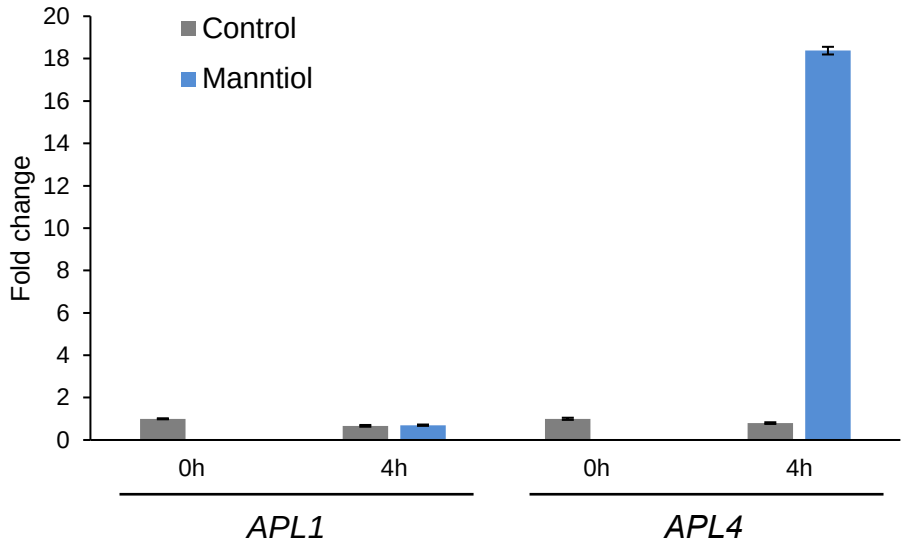


Figure 4 Leaf transcript abundance for *ISA1*, *LDA*, and *AMY3* in osmotically stressed and control leaves, determined by qPCR. Plants grown as in Thalmann et al. (2016) were harvested at the indicated time points. The *ACT2* gene was used as a reference gene.

Based on these results a knock out mutant (SALK_108632) of APL4 was obtained, and the starch content of *apl1* and *apl4* mutants was analysed during osmotic stress. As expected, *apl1* mutants accumulated less starch than wild type plants (Fig. 5). Interestingly, starch degradation was not induced by osmotic stress in this mutant. This suggests that stress-induced starch degradation only occurs if sufficient starch is present, a hypothesis that was further investigated in chapter 4. In contrast, the loss of APL4 did not affect starch metabolism in either control conditions nor during osmotic stress.

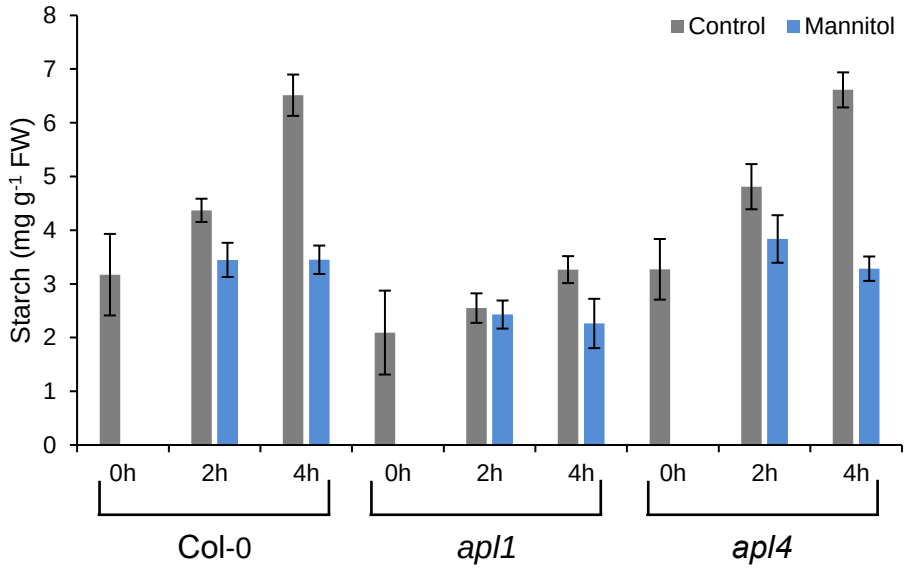


Figure 5: Leaf starch content of osmotically stressed leaves compared with controls. Values are means ± SE (n = 8). FW, fresh weight.

Further work will be necessary to understand if and how osmotic stress affects starch synthesis. In our lab we recently introgressed an *apl3* allele into Col-0 background and also created the *apl3/apl4* double mutant. Interestingly, the double mutant exhibited a delay in diurnal starch synthesis in guard cells (Horrer, 2016), indicating that some functional redundancy between the two isoforms exists. It would be interesting to analyse the responses of both *apl3* and *apl3/apl4* plants to osmotic stress.

The presence of ABRE alone is a poor indicator of stress responsiveness

Both AMY3 and BAM1 are induced by stress and their promoters contain ABREs, while BAM3 is not induced by stress and its promoter lacks ABREs. We wondered if this was a general pattern and the involvement of other starch metabolic enzymes in stress-induced starch turnover could be inferred from presence of ABREs in their promoters. To investigate this hypothesis we retrieved the 2kBp promoter of starch related enzymes from phytozome and analysed the sequences for cis-regulatory elements using plantCARE (Lescot et al., 2002). The results suggest that there is no correlation between the presence of ABREs and the stress responsiveness of a gene. No ABRE was identified in the promoter of APL4 (Table 1), yet the transcription was induced by stress (Figure 4). The promoters of BAM1, PHS1 and LDA all contained three putative ABREs, but stress increased the transcription of BAM1 much

more compared to the latter two genes. Therefore, the presence of ABREs cannot be used to predict whether a gene is induced by stress, and can only be used to infer the mechanism by which osmotic stress increases gene expression.

Gene name	AGI code	Predicted ABRE like motives
Starch degrading enzymes		
Alpha-amylases		
AMY1	AT4G25000	-
AMY2	AT1G76130	-
AMY3	AT1G69830	2
Beta-amylases		
BAM1	AT3G23920	3
BAM2	AT4G00490	2
BAM3	AT4G17090	-
BAM4	AT5G55700	-
BAM5	AT4G15210	1
BAM6	AT2G32290	2
BAM7	AT2G45880	2
BAM8	AT5G45300	-
BAM9	AT5G18670	4
Debranching enzymes		
ISA3	AT4G09020	1
LDA	AT5G04360	3
Phosphorylases		
PHS1	AT3G29320	3
PHS2	AT3G46970	1
Disproportionating enzymes		
DPE1	AT5G64860	2
DPE2	AT2G40840	1
Glucanphosphate phosphatases		
SEX4	AT3G52180	-
LSF1	AT3G01510	3
LSF2	AT3G10940	-
Dikinases		
GWD	AT1G10760	3
PWD	AT4G24450	1
Starch synthesizing enzymes		
ADGPase large subunit		
APL1	AT5G19220	-
APL2	AT1G27680	-
APL3	AT4G39210	-
APL4	AT2G21590	-

	ADGPase small subunit	
APS1	AT5G48300	-
APS2	AT1G05610	-
	Starch synthases	
SSI	AT5G24300	2
SSII	AT3G01180	-
SSIII	AT1G11720	3
SSIV	AT4G18240	-
GBSS	AT1G32900	-
	Debranching enzymes	
ISA1	AT2G39930	4
ISA2	AT1G03310	1
	Branching enzymes	
BE2	AT5G03650	-
BE3	AT2G36390	1
	Other	
PGM	AT5G51820	1
PGI	AT4G24620	3

Table 1: Number of ABREs identified in the promoters of different enzymes involved in starch turnover.

Salt stress elicits different responses than osmotic stress

In addition to mannitol and sorbitol we also tested responses of plants to two other osmolytes, PEG200 and sodium chloride (NaCl). While both sorbitol and mannitol induced starch degradation, the effect of PEG200 was less clear. Surprisingly starch content was increased by salt stress (Fig. 6). Thus, it appears that the type of osmolyte can influence the stress response. However, due to abnormally low starch content in this experiment as well as the large standard error it is difficult to draw definitive conclusions. It will be necessary to repeat this experiment to unambiguously establish that different osmolytes trigger different responses.

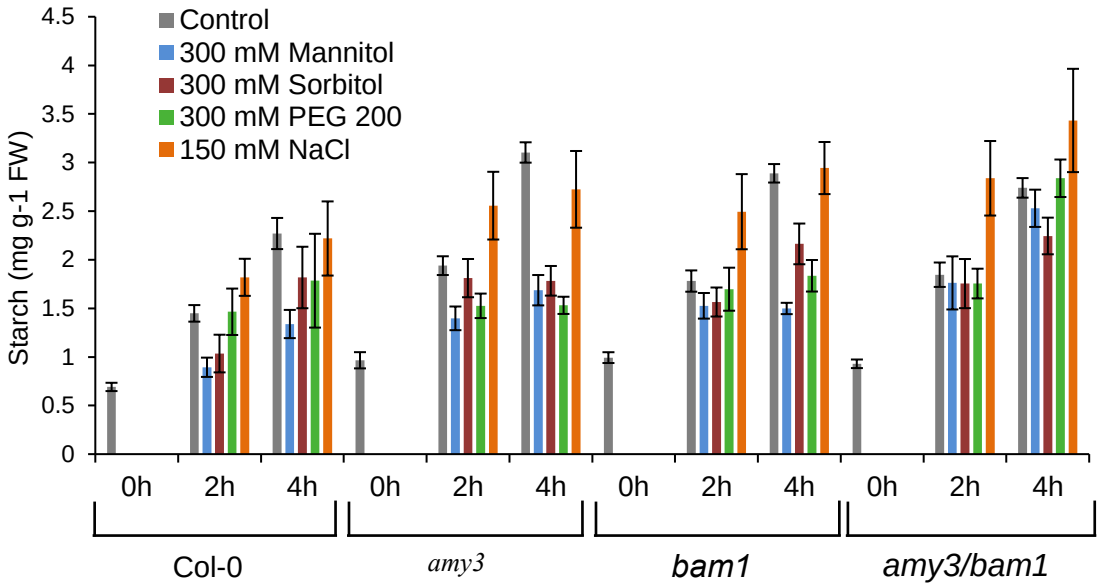


Figure 6: Leaf starch content of osmotically stressed leaves compared with controls. Values are means ± SE (n = 8). FW, fresh weight.

2 – β-amylase 1 (BAM1) degrades transitory starch to sustain proline biosynthesis during drought stress

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Synopsis: In the first chapter we showed that BAM1 plays an important role in acute osmotic stress. In this work, this observation is extended by showing that BAM1 also contributes to tolerance to prolonged mild osmotic stress. Expression of *BAM1* was induced by long-term stress (150 mM mannitol for one week) and *bam1* mutants had a starch excess phenotype under these conditions. Furthermore, during stress *bam1* plants accumulated less compatible solutes (proline, sucrose and glucose) than wild type. In contrast to the rapid changes induced by severe stress, moderate stress induced a gradual accumulation of compatible solutes over the course of several days.

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RESEARCH PAPER

β-amylase 1 (BAM1) degrades transitory starch to sustain proline biosynthesis during drought stress

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Abstract

During photosynthesis of higher plants, absorbed light energy is converted into chemical energy that, in part, is accumulated in the form of transitory starch within chloroplasts. In the following night, transitory starch is mobilized to sustain the heterotrophic metabolism of the plant. β-amylases are glucan hydrolases that cleave α-1,4-glycosidic bonds of starch and release maltose units from the non-reducing end of the polysaccharide chain. In Arabidopsis, nocturnal degradation of transitory starch involves mainly β-amylase-3 (BAM3). A second β-amylase isoform, β-amylase-1 (BAM1), is involved in diurnal starch degradation in guard cells, a process that sustains stomata opening. However, BAM1 also contributes to diurnal starch turnover in mesophyll cells under osmotic stress. With the aim of dissecting the role of β-amylases in osmotic stress responses in Arabidopsis, mutant plants lacking either BAM1 or BAM3 were subject to a mild (150 mM mannitol) and prolonged (up to one week) osmotic stress. We show here that leaves of osmotically-stressed *bam1* plants accumulated more starch and fewer soluble sugars than both wild-type and *bam3* plants during the day. Moreover, *bam1* mutants were impaired in proline accumulation and suffered from stronger lipid peroxidation, compared with both wild-type and *bam3* plants. Taken together, these data strongly suggest that carbon skeletons deriving from BAM1 diurnal degradation of transitory starch support the biosynthesis of proline required to face the osmotic stress. We propose the transitory-starch/proline interplay as an interesting trait to be tackled by breeding technologies aimingto improve drought tolerance in relevant crops.

Key words: Arabidopsis, β-amylases, drought, osmolytes, proline, transitory starch.

Introduction

Starch is a polymer of D-glucose and represents a convenient way to store carbohydrates as semi-crystalline and osmotically inert granules. The granules are mainly composed of a highly branched amylopectin polymer (70–90%), the remaining 10–30% being amylose which is much less branched (Denyer *et al.*, 2001; Zeeman *et al.*, 2002; Streb *et al.*, 2012). As a consequence of its structure, glucose units embedded in the starch granule may not be immediately available to satisfy the different demands of the organism when faced with an urgent request. The tight regulation of several enzymes

involved in starch degradation seems consistent with the need to speed up the use of starch under particular conditions, i.e. under stress (Santelia *et al.*, 2015).

Two kinds of starch, structurally indistinguishable, are found in plants: secondary and transitory starch. This physiological distinction is mainly based on different storage organs and on different rates of synthesis and degradation (Smith *et al.*, 2005). Because of its commercial relevance, secondary starch has been extensively investigated, with the aim of creating new starch structures for industrial applications (Jobling, 2004; Santelia and Zeeman, 2011; Bahaji *et al.*, 2014). Conversely, the physiology of transitory starch has become a major topic of research only recently (Zeeman *et al.*, 2007; Stitt and Zeeman, 2012), with increasing evidence of the involvement of transitory starch metabolism in response to stress (Hummel *et al.*, 2010; Valerio *et al.*, 2011; Prasch *et al.*, 2015; Santelia *et al.*, 2015).

Due to their sessile nature, plants have to cope not only with rapid and daily environmental changes, but they must also balance the energy needed for growth with the energy required for stress responses. Starch biosynthesis is tightly correlated with photosynthesis, another process strongly affected by the environment. In the model plant *Arabidopsis thaliana*, half of the photoassimilates produced by the Calvin–Benson cycle during the day are typically exported to the cytosol to supply carbon skeletons for anabolic or catabolic processes, whereas the remaining half is retained in the chloroplast for transitory starch biosynthesis (Zeeman and ap Rees, 1999). Under normal growth conditions, the export of organic carbon is mediated by two different transport mechanisms which operate at different times of the diurnal cycle. During the day, photoassimilates mainly reach the cytosol via the triose phosphate/phosphate translocator (TPT) (Flügge, 1999) whereas, during the night, β-maltose (the major product of starch degradation) and glucose are exported to the cytoplasm via the maltose (MEX1) (Nittylä *et al.*, 2004) and glucose (GLT and GT) (Cho *et al.*, 2011; Flügge *et al.*, 2011) transporters, respectively.

β-Amylases are the only enzymes that produce β-maltose, thereby connecting starch degradation in chloroplasts with sugar metabolism in the cytoplasm. Several β-amylases are encoded by the Arabidopsis genome (Lloyd *et al.*, 2005). BAM3 is a major, catalytically active β-amylase that is necessary for nocturnal starch degradation under physiological conditions. Conversely, BAM1 is little or not involved in this process (Fulton *et al.*, 2008; Kötting *et al.*, 2010). However, in response to drought or salt stress, BAM1 becomes a predominant β-amylase of leaves and is required for starch breakdown in mesophyll cells (Valerio *et al.*, 2011; Monroe *et al.*, 2014).

Water stress has severe negative impacts on plant growth and productivity (Cattivelli *et al.*, 2008; Rockström and Falkenmark, 2010; Osakabe *et al.*, 2014). A common trait of many plants affected by drought or salinity stress is the accumulation of osmoprotectants such as proline, glycine betaine, and sugar alcohols (Szabados and Savouré, 2009; Liang *et al.*, 2013). Proline accumulation occurs at very high levels when plants experience conditions of low water potential. Proline concentration can increase up to 100-fold compared with control conditions (Verbruggen and Hermans, 2008; Szabados and Savouré, 2009). However, proline not only functions as an osmoprotectant, but it can also scavenge reactive oxygen species (ROS) efficiently, thus protecting the cell from oxidative damage (Matysik *et al.*, 2002, Bartels and Sunkar, 2005).

In plants, proline synthesis occurs both in the cytosol and in the chloroplast, whereas degradation only occurs in mitochondria. Carbon skeletons for proline biosynthesis are provided by primary metabolism through the glutamate pool. Whether starch degradation is involved in this process is currently unknown.

To investigate the possible interplay between transitory starch and proline metabolism under drought stress, the response to 150 mM mannitol treatments of two single T-DNA insertion mutants, *bam1* and *bam3*, and wild-type plants was studied and compared. The findings strongly suggest that, in the drought stress response of Arabidopsis, BAM1 and not BAM3 is the major player in starch degradation in the light, a metabolic pathway that provides carbon skeletons for the biosynthesis of sucrose and proline to counteract both osmotic stress and oxidative damage.

Materials and methods

Plant material and growth conditions

Wild-type, T-DNAs, and *BAM1* promoter::GUS plants of *Arabidopsis thaliana* (ecotype Columbia, Col-0) were hydroponically grown at a constant temperature of 22 °C, under a 12/12 h light/dark cycle with a photosynthetic photon flux density of 110 μmol m^{-2} s^{-1}, as described in Valerio *et al.* (2011). The GUS line and insertion sites of the T-DNA in *bam1* (SALK_039895) and *bam3* (CS92461) mutants had already been analysed (Fulton *et al.*, 2008; Valerio *et al.*, 2011).

Stress conditions

To analyse the response of Arabidopsis plants to drought further, previously tested conditions (300 mM mannitol for up to 8 h; Valerio *et al.*, 2011) were changed in order to obtain a mild (150 mM mannitol) and prolonged (up to 7.5 d) osmotic stress. Mild osmotic stress was applied to 28/31-d-old plants (with 3/4 d of stratification time at 4 °C in darkness excluded), 1 h after switching on the light. Treated plants were transferred to a freshly prepared hydroponic medium supplemented with 150 mM mannitol. If not differently specified, plants were harvested either at the end of the light period (12 h light) or at the end of the dark period (12 h dark), every 12 h for a maximum of 7.5 d after the beginning of the treatment (DAT). Samples were immediately frozen in liquid nitrogen and stored at −80 °C for analysis.

GUS staining

Histochemical GUS staining was performed as described in Valerio *et al.* (2011). For each condition and for each time point, three independent transgenic plants were analysed. Control and treated (150 mM mannitol) plants were collected every day during the experiment, always at the end of the 12 h light period. Stained plants were examined by bright-field microscopy using a Nikon Eclipse 90-I microscope. The images show representative plants and leaves.

Determination of water loss

The loss of water from the leaves was determined as the ratio between the dry weight (DW) and the fresh weight (FW), measured on single

plants collected after 12 h of light and 12 h of dark, under control or stress conditions, during a 6 d experiment. FW was scored immediately after excision and DW was determined after incubation at 80 °C for 24 h. Five independent biological replicates were analysed.

Quantification of starch and soluble sugars

Quantification of starch and soluble sugars were carried out on whole rosette leaves of 3–5 plants for each experimental point. Starch was quantified on bleached leaves as described in Smith and Zeeman (2006). Quantification of sucrose, glucose, and maltose was performed as described in Egli *et al.* (2010) on freeze-dried supernatants obtained after extracting with 80% ethanol for 15 min at 80 °C. Three independent biological replicas were analysed.

Lipid peroxidation assay

Oxidative damage was estimated by measuring total lipid peroxidation using the 2-thiobarbituric acid (TBA) assay, as described in Guidi *et al.* (1999). Briefly, about 200 mg of leaves were powdered in liquid nitrogen, before being vigorously mixed with 3 vols of 0.1% (w/v) trichloroacetic acid (TCA). Samples were centrifuged and 0.5 ml of each supernatant was transferred into a screw cap tube in the presence of 2.0 ml 20% (w/v) TCA and 1.5 µl 0.5% (w/v) TBA. Following a 30 min incubation at 90 °C, the reaction was stopped by placing the tubes in a bath of ice water. Samples were centrifuged and the absorbance of the supernatants was monitored at 532 nm, subtracting the non-specific absorption at 600 nm. The amount of MDA–TBA complex was calculated from the extinction coefficient 155 mM^{-1} cm^{-1}. Three independent biological replicas were analysed.

Proline quantification

Samples stored at −80 °C were ground in liquid nitrogen and the free proline content was measured as described by Bates *et al.* (1973). Briefly, 1.2 ml of 3% 5-sulphosalicylic acid was added to 50 mg of powdered leaves. Samples were centrifuged and appropriate volumes of supernatant were transferred into clean tubes and brought to a final volume of 1 ml with water, and then mixed with 1 ml of glacial acetic acid and 1 ml of 2.5% ninhydrin reagent Samples were incubated at 90 °C for 1 h, cooled on ice, combined with an equal volume of toluene, and mixed vigorously. Following phase partitioning, the absorbance of the upper phase was monitored at 520 nm. The calibration curve was prepared using different proline concentrations as standard. From 3–4 independent biological replicates were analysed.

Results

Mild osmotic stress induces BAM1 *promoter activity*

To understand the activation of *BAM1* in response to mild osmotic stress better, the activity of *GUS* in Arabidopsis plants stably transformed with the *BAM1* promoter controlling the GUS reporter gene (*BAM1promoter::GUS plants*) was examined. Adult plants were exposed to 150 mM mannitol and collected every day for one week.

As previously reported in Valerio *et al.* (2011), in the absence of stress, GUS activity of *BAM1promoter::GUS* plants was mainly confined to guard cells (see Supplementary Fig. S1 at *JXB* online) and almost absent from mesophyll cells (Fig. 1, right panel). Under mild osmotic stress, a slight increase in the promoter activity of *BAM1* had already appeared at the beginning of the stress, albeit confined to leaf veins (Fig. 1A, B, left panel). Upon prolonged stress, GUS activity spread to mesophyll cells, first in young leaves and then throughout the whole rosette (Fig. 1C–E, left panel).

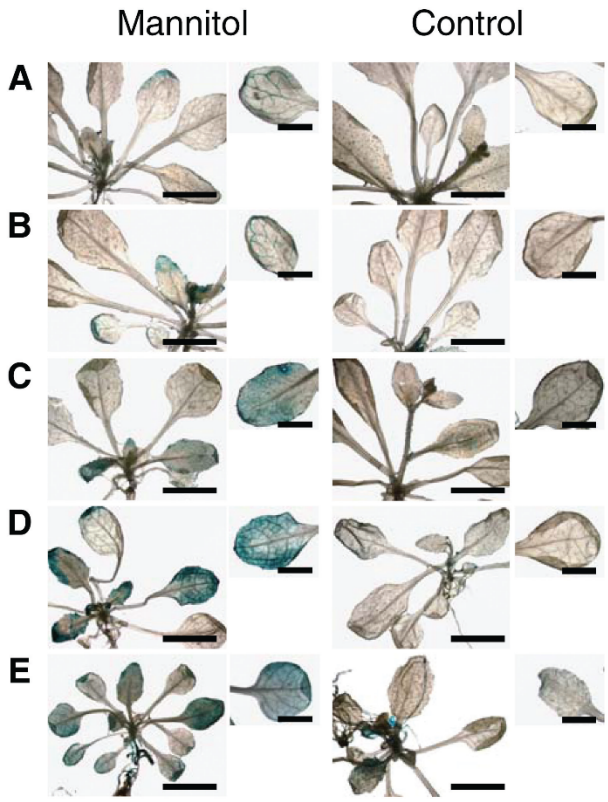

Fig. 1. Activity of BAM1promoter::GUS under control conditions and in response to 150 mM mannitol treatment. Plants were grown under a 12/12 h light/dark cycle and osmotic stress was applied 1 h after the beginning of the light period. Plants were collected at the end of the light period. GUS activity was measured at 0.5 DAT (A); 1.5 DAT (B); 3.5 DAT (C); 6.5 DAT (D), and 7.5 DAT (E). Scale bar=1 cm. Inset: magnification of a single leaf. Scale bar=0.5 cm.

Water loss in response to stress

β-amylase 3 (BAM3) is the major isoform responsible for transitory starch degradation at night (Lao *et al.*, 1999; Fulton *et al.*, 2008). To get insights into the role of BAM1 in starch degradation in response to osmotic stress, *bam3* T-DNA mutant plants were also analysed. Dehydration rates of *bam1*, *bam3*, and wild-type plants in response to 150 mM mannitol were determined (see Supplementary Fig. S2 at *JXB* online). The data obtained did not show statistically significant differences among the three genotypes, neither in response to stress nor in control conditions (see Supplementary Table S1 at *JXB* online). The similar decrease in water content observed in the three genotypes during the whole experiment, allows a comparison between genotypes of data expressed on a FW basis.

Starch content at the end of the light period

To investigate the involvement of BAM3- and BAM1-dependent starch degradation pathways in response to drought stress, the starch content was measured in leaves after 12 h light, before and after the mannitol treatment (Fig. 2; see Supplementary Fig. S3 at *JXB* online).

Consistent with the predominant role of BAM3 in transitory starch degradation (Fulton *et al.*, 2008), under control

growth conditions *bam3* plants showed the well-known *s*tarch *e*xcess (*sex*) phenotype, characterized by small plants with a high starch content (~3-fold higher compared with wild-type plants) (Fig. 2). Conversely, compared with wild-type plants, *bam1* mutant plants did not show any significant change in starch concentration (Fig. 2; see Supplementary Table S2 at *JXB* online), again in agreement with the literature (Fulton *et al.*, 2008).

In response to osmotic stress, the ratio in starch content between *bam3* and wild-type samples suddenly decreased from ~3 (in the absence of mannitol) to ~2 (in the presence of mannitol), remaining roughly constant throughout the experiment (Fig. 2). On average, the amount of starch contained in *bam3* plants at the end of the day was reduced by ~50 µmol glucose equivalents g^{-1} FW as a consequence of the stress. Although with different timing, an opposite behaviour was observed in *bam1* plants. During the first three days of the experiment, starch content in *bam1* plants remained similar to the wild type, but doubled wild-type levels from the fourth day onwards (Fig. 2). An average increase of ~50 µµmol glucose equivalents g^{-1} FW was calculated.

Starch content at the end of the night period

To analyse the involvement of β-amylases on transitory starch turnover in response to drought further, the starch concentration was also measured at the end of the night period (12 h dark), before and after mannitol treatment (Fig. 3). As expected, under control condition, wild-type and *bam1* plants did not differ in their starch content while *bam3* plants confirmed the *sex* phenotype (Fig. 3; see Supplementary Table S3 at *JXB* online) (Fulton *et al.*, 2008).

High levels of starch were maintained in *bam3* mutants in the first two days of the experiment (Fig. 3). Conversely, *bam1* plants rapidly responded to 150 mM mannitol with an increase in starch concentration that, within the first two days of the experiment, made them closer to *bam3* than to wild-type plants. Later in the experiment (from 3–6 DAT) no significant differences were observed among the three genotypes in response to 150 mM mannitol (Fig. 3).

Lipid peroxidation

A common effect of osmotic stress is the accumulation of free oxygen radicals (Aranjuelo *et al.*, 2011; Wilhelm and Selmar, 2011) leading to oxidation of unsaturated fatty acids and membrane damage (Hernandez *et al.*, 1993; Fadzilla *et al.*, 1997). Lipid peroxidation induced by osmotic stress was evaluated as the malondialdehyde (MDA) concentration on *bam1*, *bam3*, and wild-type plants treated with 150 mM mannitol. The exposure to the osmotic stress increased the MDA concentration in all genotypes in a time-dependent manner (Fig. 4; see Supplementary Table S4 at *JXB* online). However, only *bam1* samples collected at 4.5 DAT showed a ~2-fold increase in MDA concentration compared with the wild type, suggesting that BAM1 is an essential component of the Arabidopsis response to the oxidative damage caused by the osmotic stress.

Proline content

Proline is considered a compatible osmolyte and its accumulation in response to different stresses has been reported in several plant species (Szabados and Savouré, 2009). In order to test whether proline accumulation in osmotically stressed Arabidopsis plants might depend on the activity of β-amylases, the proline concentration was measured in rosette leaves of wild-type, *bam1*, and *bam3* plants subject to 150 mM mannitol treatments (Fig. 5). In the absence of stress, similar proline concentrations (~0.67 µµmol g^{-1} FW) were measured in the three genotypes and no significant differences were observed until 2.5 DAT (Fig. 5; see Supplementary Table S5

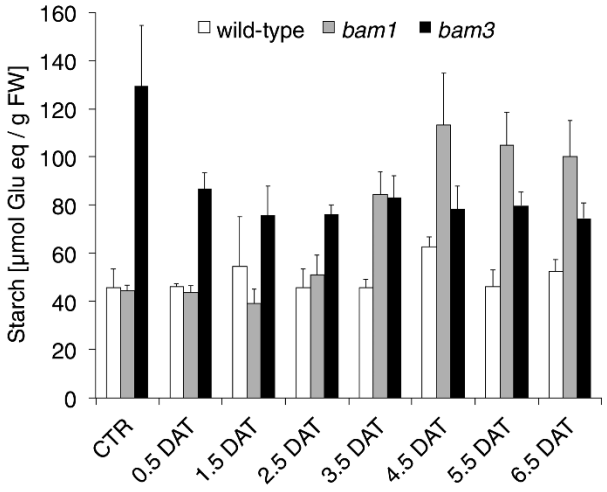

Fig. 2. Starch content in wild-type, *bam1*, and *bam3* plants measured after 12 h of light in response to drought stress. Twenty-eight/31-d-old hydroponically grown plants were expose to 150 mM mannitol 1 h after switching on the light. Wild-type, *bam1*, and *bam3* plants were collected after 12 h of light before and after mannitol treatment. Values are the means ±SD (*n*=3 independent biological replicates).

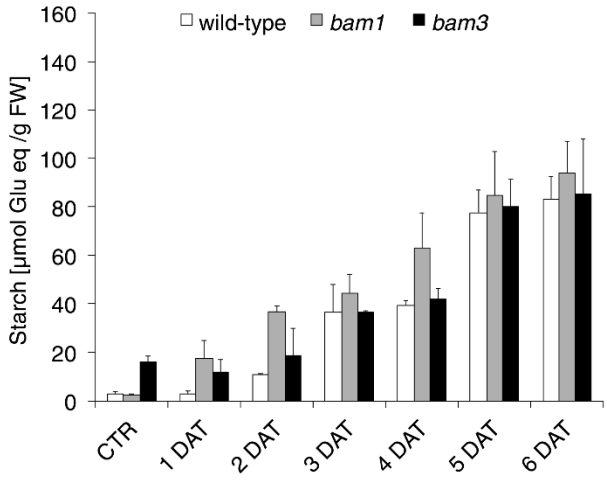

Fig. 3. Starch content in wild-type, *bam1*, and *bam3* plants after 12 h of darkness in response to drought stress. Twenty-eight/31-d-old hydroponically grown plants were exposed to 150 mM mannitol 1 h after switching on the light. Wild-type, *bam1*, and *bam3* plants were collected after 12 h of darkness before and after mannitol treatment. Values are the means ±SD (*n*=3 independent biological replicates).

at *JXB* online). At 3.5 DAT, both *bam1* and *bam3* mutants showed less proline accumulation with respect to the wild type. However, at later time points, only the *bam1* mutant showed a limited accumulation of proline, while *bam3* plants recovered the same proline concentration as wild-type plants (Fig. 5).

Interestingly at 6.5 DAT, the lower proline content of the *bam1* mutant with respect to the wild-type (and *bam3* plants) corresponded to ~37 µmol proline g^{-1} FW (Fig. 5). Considering that the same mutant at the same time point accumulated a surplus of ~48 µmol glucose equivalents g^{-1} FW (Fig. 2), it seems reasonable that impaired starch degradation was the reason for the failure in proline accumulation.

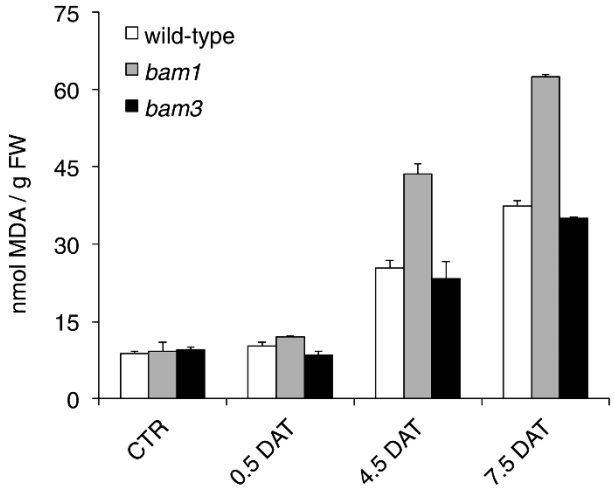

Fig. 4. Degree of lipid peroxidation in wild-type, *bam1*, and *bam3* plants exposed to osmotic stress. Lipid peroxidation was measured using the TBA assay in wild-type, *bam1*, and *bam3* plants before and after the 150 mM mannitol treatment. Plants were collected after 12 h light and different lengths of treatment. Values are the means ±SD (n=3 independent biological replicates).

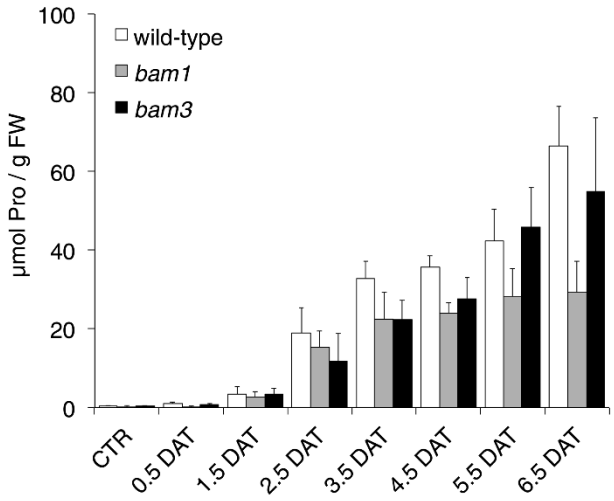

Fig. 5. Proline content in wild-type, *bam1*, and *bam3* plants in response to drought stress. Proline concentration was measured in whole rosettes of 28/31-d-old wild-type, *bam1*, and *bam3* plants. Plants were collected after 12 h of light before and after 150 mM mannitol treatment. Values are the means ±SD (n=3–4 independent biological replicates).

Soluble sugars

Sucrose, maltose, and glucose concentrations were measured in wild-type, *bam1*, and *bam3* plants in response to 150 mM mannitol both after 12 h of light and after 12 h of dark (Fig. 6; see Supplementary Table S6 at *JXB* online). Under control conditions, the concentration of soluble sugars in all genotypes at the end of the day or at the end of the night, resembled the values already reported in the literature (Fulton *et al.*, 2008; Hummel *et al.*, 2010). Glucose was higher than sucrose, which was much higher than maltose, and all three sugars appeared to be more concentrated at the end of the day than at the end of the night.

Similar to what was observed for transitory starch (Fig. 3), during the osmotic stress experiment, soluble sugar concentrations measured at the end of the night were essentially similar among the genotypes (Fig. 6, right panels), with the only exception being maltose in the *bam3* mutant at 1 DAT, which was more concentrated than in the wild type (Fulton *et al.*, 2008). By contrast, at the end of the day, *bam1* plants showed a general decrease in sucrose, glucose, and maltose concentrations with respect to both wild-type and *bam3* plants (Fig. 6, left panels). By comparison with wild-type plants at 5.5 DAT, the absence of BAM1 led to a decrease of ~2.8 µmol sucrose g^{-1} FW, ~5.9 µmol glucose g^{-1} FW, and ~55 nmol of maltose g^{-1} FW.

Discussion

Plants are sessile organisms with a metabolism that essentially depends on light and needs to be continuously adapted to environmental changes. A fundamental aspect of this adaptation consists of the circadian cycles of diurnal synthesis and nocturnal degradation of transitory starch that allow plants to harmonize with the natural rhythm of light availability (Stitt and Zeeman, 2012). Nocturnal degradation of transitory starch sustains basal metabolism and the reallocation of organic carbon in the absence of an external input of energy. On top of that, under stress conditions, plants need to redirect transitory carbon fluxes in order to fuel stress responses, a decision that often implies detrimental effects on growth. As far as transitory starch is concerned, its degradation and use of the resulting carbon units for stress responses involve a large set of enzymes, including β-amylases.

With the aid of *bam3* and *bam1* knock-out mutants (Fulton *et al.*, 2008; Valerio *et al.*, 2011), we have investigated the relative contribution of BAM1 and BAM3 to transitory starch degradation in response to mild and prolonged osmotic stress. BAM3 is required for nocturnal starch degradation under physiological conditions (Fulton *et al.*, 2008), while BAM1 is dispensable for transitory starch degradation in the absence of stress, but is activated by drought stress at the transcriptional level and post-translationally activated by reduced thioredoxins (Sparla *et al.*, 2006; Valerio *et al.*, 2011). Under control growth conditions, the rosette leaves of *bam3* mutants contained high levels of starch during the whole day, which were always higher than the wild-type plants. Under osmotic stress, the starch levels of *bam3* plants

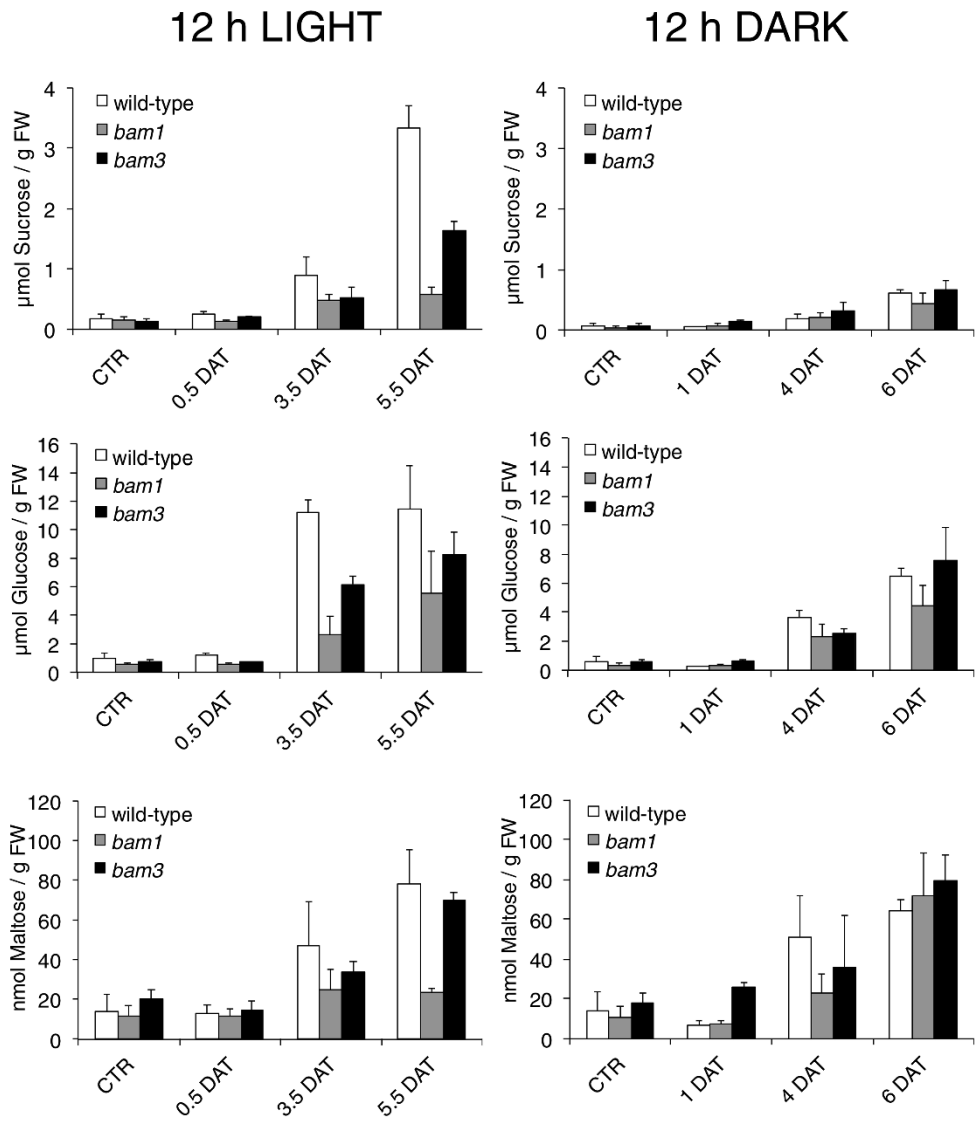

Fig. 6. Sucrose, glucose, and maltose content in wild-type, *bam1*, and *bam3* plants measured after 12 h of light and aftert 12 h of darkness in response to drought stress. Hydroponically grown Arabidopsis plants were exposed to 150 mM mannitol 1 h after switching on the light. Whole rosettes of wild-type, *bam1*, and *bam3* plants were collected after 12 h of light (left panels) and 12 h of darkness (right panels) before and after 150 mM mannitol treatment. Values are the means ±SD (n=3 independent biological replicates).

suddenly decreased, particularly during the light and became closer to wild-type levels. Different from *bam3*, under control growth conditions, the levels of leaf starch in *bam1* mutants were similar to the wild-type plants, in agreement with the notion that BAM1 is confined to guard cells until plants start to flower (Valerio *et al.*, 2011; Prasch *et al.*, 2015). However, in response to osmotic stress, BAM1 also appears in mesophyll cells and the starch content in *bam1* mutants increased, particularly so at the end of the light and after several days of stress. In conclusion, a mild, prolonged osmotic stress caused a decrease in daylight starch in plants with no BAM3 and, conversely, an increase in daylight starch in plants with no BAM1, suggesting that BAM1 is involved in daylight starch degradation upon stress. This hypothesis fits with both the induction of the BAM1 promoter by the osmotic stress and the redox regulation of BAM1 that favours its activity in the light (Sparla *et al.*, 2006; Valerio *et al.*, 2011).

Plants have evolved several different mechanisms to respond to limited water availability and proline accumulation has long been reported to be a part of the drought-stress response (Szabados and Savouré, 2009). The main pathway of proline biosynthesis derives from glutamic acid and it can occur both in the cytosol and the chloroplast. Under stress conditions, however, the plastidial pathway of proline biosynthesis may prevail as a result of the re-localization of Δ^1-pyrroline-5-carboxylate synthetase (P5CS1) into chloroplasts (Székely *et al.*, 2008). P5CS1 catalyses the limiting step of proline biosynthesis and its role in proline accumulation in water-stressed plants is recognized (Székely *et al.*, 2008). Although each of the three genotypes investigated in our study (*bam1*, *bam3*, and Col-0) accumulated proline under osmotic stress, the proline concentration of *bam1* mutants did not reach the same levels as those reached by wild-type and *bam3* plants. The lack of adequate proline accumulation in *bam1* mutants

correlated with a more severe oxidative stress in these plants, as judged by the extent of lipid peroxidation. Moreover, lower proline levels in *bam1* plants went together with lower concentrations of sucrose, glucose, and maltose and, as discussed above, higher levels of starch. Following several days of stress, the starch content in *bam1* plants at the end of the photosynthetic period exceeded wild-type levels by about 50 µmol glucose equivalents g^{-1} FW. To put this value into context, proline accumulation in these same plants and under the same conditions was lower than in wild-type plants by 37 µmol g^{-1} FW, while soluble sugars (sucrose and glucose) decreased by 12 µmol hexoses g^{-1} FW. Based on these numbers, the reason why *bam1* plants had less proline and soluble sugars upon stress may well be that the carbon skeletons required to make these osmolytes are stuck into starch granules and, as such, are not available. Since BAM1 is suggested to play a role in starch degradation under these conditions, it makes sense that its absence has more dramatic effects during the day, when BAM1 is redox-activated and P5CS1 is sufficiently concentrated (Hayashi *et al.*, 2000; Székely *et al.*, 2008) to catalyse the metabolic flux leading to proline.

Although the whole pathway connecting the degradation of transitory starch with the biosynthesis of proline still remains to be discovered, the results presented here strongly suggest a link between these two metabolic pathways and suggest a role for BAM1 in this context. Our results suggest that a mild osmotic stress stimulates starch turnover in the light through the activation of BAM1, both at the transcriptional and post-translational level. Indeed, BAM1 activity is strictly redox-regulated and, since it requires thioredoxin f to be highly reduced, BAM1 is predicted to be more active under photosynthetic conditions (Sparla *et al.*, 2006). Based on correlative observations, we propose that maltose derived from BAM1 degradation of starch upon stress sustains the biosynthesis of proline (and soluble sugars) thereby alleviating the oxidative stress. Since water availability is a major constraint for modern agriculture, the efforts in selecting crops with better water use efficiency should take into account this link between starch and proline metabolism.

Supplementary data

Supplementary data can be found at *JXB* online.

Figure S1. Activity of *BAM1promoter::GUS* under control conditions and in response to 150 mM mannitol.

Figure S2. Loss of water in wild-type, *bam1*, and *bam3* plants exposed to 150 mM mannitol.

Figure S3. Starch content in wild-type, *bam1* and *bam3* plants qualitatively evaluated with Lugol staining.

Table S1. *P* value from Student's *t* tests performed on loss of water.

Table S2. *P* value from Student's *t* tests performed on starch concentration quantified after 12 h of light.

Table S3: *P* value from Student's *t* tests performed on starch concentration quantified after 12 h of dark.

Table S4. *P* value from Student's *t* tests performed on degree of lipid peroxidation.

Table S5. *P* value from Student's *t* tests performed on proline concentration.

Table S6. *P* value from Student's *t* tests performed on sucrose, glucose, and maltose concentrations quantified after 12 h of light and 12 h of dark.

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3 – Evidence for post-translational modification of BAM1 during stress

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Unpublished results

Synopsis: BAM1 is known to undergo post-translational modifications, such as the formation of a disulphide bridge (inhibitory) and phosphorylation by the protein Shaggy-like kinase ATSK13 (possibly stimulatory). We tried to assess whether these or other modifications contributed to the regulation of BAM1 during osmotic stress. We initially saw a shift of electrophoretic mobility of BAM1 of ~10 kDA in response to osmotic stress. This shift was independent of ABA and not caused by the action of ATSK13. However, this shift was only reproducible for about 8 months during the winter 2014/15 and could not be observed afterwards. We reanalysed old samples and could show that the lack of band shift was not an artefact of the blotting process. It remains unclear why the shift can no longer be observed.

Introduction

Post-translational protein modifications

After being translated by ribosomes, proteins can be subjected to multitude of covalent modifications. Over three hundred different such post-translational modifications have been identified, including phosphorylation, acetylation, glycosylation, ubiquitinylation, and attachment of lipids (Mann and Jensen, 2003; Nørregaard Jensen, 2004). These modifications can drastically alter protein properties such as stability, activity, partial degradation, and interaction with other proteins. Although most investigations of post-translational modifications have been conducted in animals, studies in plants uncovered similar diversity of post-translational modifications (Kwon et al., 2006). Interestingly, a number of starch metabolic enzymes have been shown to be regulated by post-translational modifications, for example by protein phosphorylation, redox-regulation or partial proteolysis.

Phosphorylation is a very common post-translational modification, which often affects the activity of the target protein. Due to its reversibility, it plays a major role in signalling transduction. A large number of proteins involved in starch metabolism have been found to be phosphorylated (Kötting et al., 2010), although neither the significance of phosphorylation in starch metabolism nor the responsible kinases have been identified so far.

A second important post-translational modification is redox regulation which involves linking the thiol groups (reduced) of cysteine residues, forming disulphide bridges (oxidised). Alternatively, a single thiol group can also be modified by glutathionylation or nitrosylation (Santelia et al., 2015). Redox regulation was shown to be of fundamental importance for control of starch synthesis by regulating the activity of AGPase through the formation of an inhibitory disulphide bridge. Replacement of cysteine^{81} (cys^{81}) with a serine results in a constitutively activated AGPase, leading to strong perturbations of leaf carbohydrate metabolism (Hädrich et al., 2012). Many other enzymes involved in starch metabolism have also been reported to be redox-regulated (Kötting et al., 2010; Glaring et al., 2012; Santelia et al., 2015), although the role of this modifications has not yet been studied *in vivo*.

Another important modification is ubiquitinylation, which often induces protein degradation by delivering the modified protein to the 26S proteasome (Vierstra, 2009). While the proteasome often degrades proteins completely, a protein may also be degraded only partially, altering the properties of the remaining protein fragment. It was shown that the sweet potato (*Ipomoea batatas* Lam. cv Tainong) phosphorylase 1 (PHO1) was targeted to the proteasome in response to heat stress. The partial

degradation resulted in a removal of a sterically hindering protein domain and elevated the activity of the enzyme (Lin et al., 2012).
Furthermore, post-translational modification of proteins has been shown to be involved in response to other abiotic stresses (Mazzucotelli et al., 2008). Due to the role of post-translational modifications in both stress response and starch metabolism, we investigated whether stress-induced starch degradation is also controlled after translation.

BAM1 is subject to posttranslational modifications

The BAM1 protein can form an intramolecular disulphide bridge between two cysteine residues, cys^{73} and cys^{511}, which inhibits its enzymatic activity (Sparla et al., 2006). This inhibition is fully reversible and BAM1 activity is restored by reducing the disulphide bridge. Consistent with the observed role of BAM1 in starch degradation during the day, reducing conditions are thought to occur in illuminated chloroplasts, as electrons produced by the photosynthetic electron transport chain can be transferred to thioredoxins which mediate the reduction of disulphide bridges of target proteins (Lemaire et al., 2007; Montrichard et al., 2009).
Furthermore, BAM1 has been found to be phosphorylated in several phosphoproteomic studies (Reiland et al., 2009; Wang et al., 2013; Lohrig et al., 2009; Nakagami et al., 2010). The serine phosphorylation occurs at a SP-XX-SP motif which is known to be a consensus site of glycogen synthase kinase 3 (GSK3)-like kinase family (de la Fuente van Bentem et al., 2008). The Arabidopsis genome contains 10 genes encoding GSK3-like kinases (Jonak and Hirt, 2002). Interestingly, one isoform, ATSK13, is induced by osmotic stress (Charrier et al., 2002), and thus represents a potential candidate for BAM1 phosphorylation. Indeed recombinant ATSK13 was able to phosphorylate BAM1 (Horrer, 2016). The influence of this phosphorylation has so far not been established definitively. While ATSK13 mediated phosphorylation does not affect the activity of recombinant BAM1 protein *in vitro*, starch degradation in guard cells was impaired in the *atsk13* mutant, mimicking the phenotype of *bam1* mutants (Horrer, 2016).
When characterising *bam1* mutants, Fulton et al., (2008) noted that BAM1 appeared as two distinct bands in western blots of wild-type plants. The more prominent upper band was found at the expected molecular weight of ~ 70 kDa, while the lower, fainter band, was found at ~ 60 kDA. Both bands were absent in *bam1* mutants, indicating that the lower band was not due to unspecific binding of the antibody. The presence of two distinct BAM1 protein species can be explained by post-translational modifications which influence the electrophoretic mobility.

Given the clear evidence for post-translational modification of BAM1 in particular we focused our investigation on the changes of this protein during osmotic stress.

Material and methods

Plant growth and osmotic stress induction

Plants were grown in hydroponics as described in chapter 1. Osmotic stress was induced as described in chapter 1 by adding either a 300mM mannitol solution or a 300 mM sorbitol solution. The following genotypes were used:

Allele	Line	Publication
bam1	SALK_039895	(Sparla et al., 2006)
nced3	GABI_129B08	(Wan and Li, 2006)
areb1areb2abf3	SALK_002984	(Yoshida et al., 2010)
	SALK_069523	
	SALK_096965	
atsk13	-	

Extraction and immunodetection of BAM1 and AMY3 proteins

Rosettes of stressed and control plants were harvested and proteins were extracted as described in chapter 1. Gel and western blot were made as described in chapter 1. Pictures were acquired either as in chapter 1 or with a Fusion FX machine (Vilber Lourmat, Germany). Quantification of band intensities was conducted using the densitometry feature of ImageJ software.

Purification of recombinant BAM1-cTP from E.coli

A pET28a+ vector (Invitrogen) containing *BAM1* cDNA (excluding the predicted transit peptide containing amino acids 1-90) fused in frame with a N-terminal histidine tag was received from David Seung (Seung et al., 2013). Recombinant BAM1 was expressed in *Escherichia coli* BL21 (DE3) CodonPlus cells (Stratagene, Basel, Switzerland). Cells were lysed and BAM1 protein was purified from the lysate using Ni^{2+}-nitrilotriacetic acid-agarose affinity chromatography as described previously (Kötting et al., 2005; Santelia et al., 2011).

Results

Osmotic stress increases the intensity of the lower band

We observed that the intensity of the lower band reported by (Fulton et al., 2008) dramatically increased if plants were exposed to osmotic stress (Fig.1). Both bands were absent in *bam1* mutants showing that both correspond to BAM1 protein. Although the lower band was barely visible at the beginning, its intensity increased during the

course of the osmotic stress and it became the major band after 8 hours. As the SDS-PAGE was run under reducing conditions (2mM DTT), the band shift is not due to the formation of an intramolecular disulphide bridge or glutathionylation.

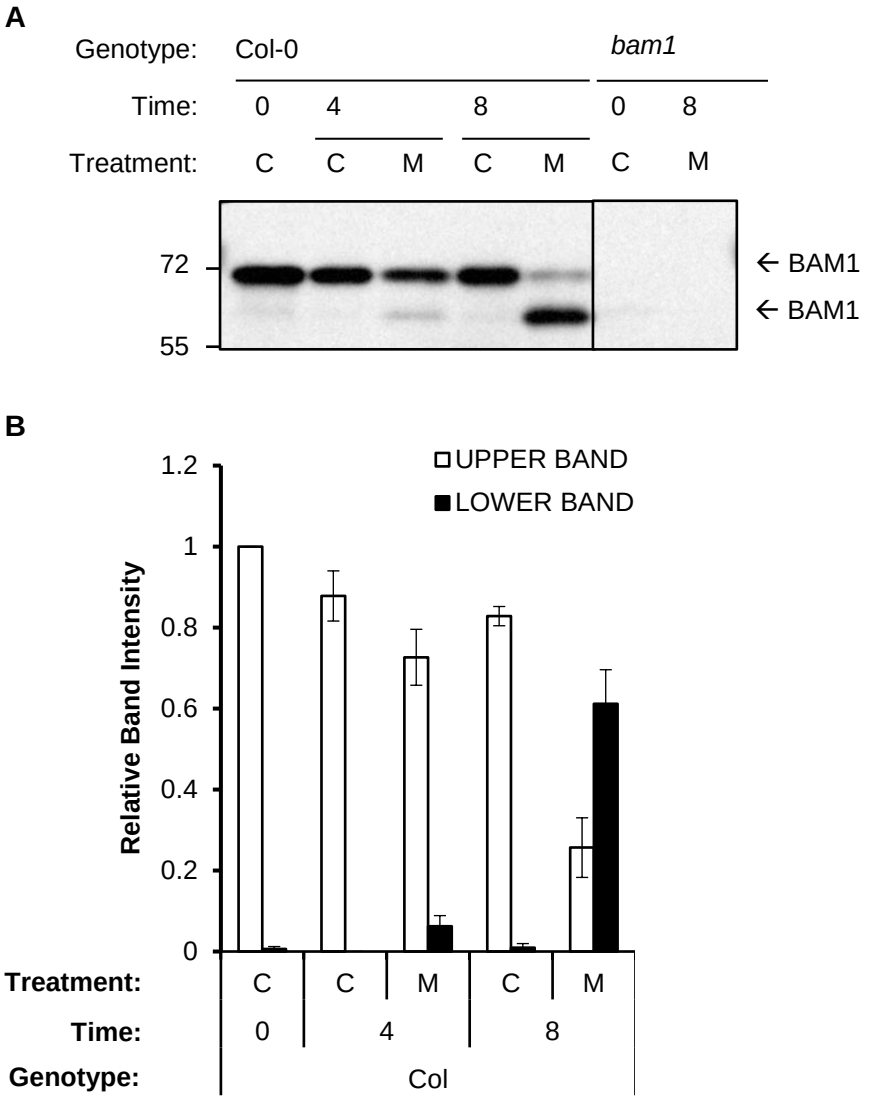
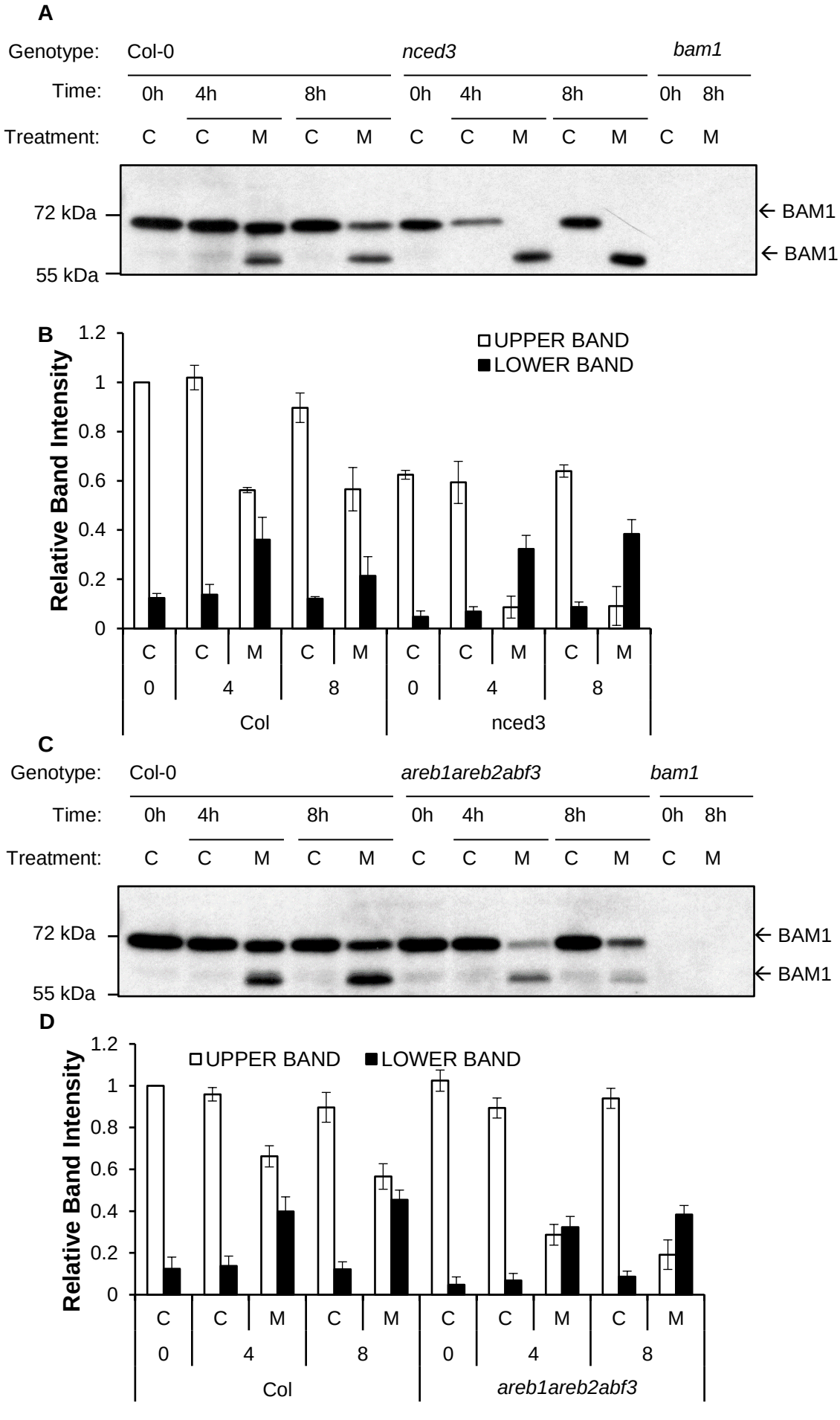


Figure 1: (A) Immunodetection of BAM1 protein in wild-type leaves after osmotic stress (Mannitol) treatment. Total protein was extracted from rosettes of hydroponically grown plants at the indicated time points.

(B) BAM1 protein quantification densitometry analysis was used to quantify band intensities of three independent biological replicates.

The change in electrophoretic mobility is ABA-independent

We investigated whether the observed shift was dependent on ABA, by examining the behaviour of BAM1 in the ABA deficient *nced3* mutant as well as in the ABA signalling mutant *areb1/areb2/abf3* (Fig. 2). The band shift evidently still occurred in both mutants and is therefore not dependent on ABA. Interestingly, the upper band disappeared completely during osmotic stress in the *nced3* mutant.


A
Genotype:
Col-0
nced3
bam1
Time:
0h
4h
8h
0h
4h
8h
0h
8h
Treatment:
C
C
M
C
M
C
C
M
C
M
C
M
72 kDa
55 kDa
← BAM1
← BAM1
B
UPPER BAND
LOWER BAND
Relative Band Intensity
1.2
1
0.8
0.6
0.4
0.2
0
C
C
M
C
M
C
C
M
C
M
0
4
8
0
4
8
Col
nced3
C
Genotype:
Col-0
areb1areb2abf3
bam1
Time:
0h
4h
8h
0h
4h
8h
0h
8h
Treatment:
C
C
M
C
M
C
C
M
C
M
C
M
72 kDa
55 kDa
← BAM1
← BAM1
D
UPPER BAND
LOWER BAND
Relative Band Intensity
1.2
1
0.8
0.6
0.4
0.2
0
C
C
M
C
M
C
C
M
C
M
0
4
8
0
4
8
Col
areb1areb2abf3

The change in electrophoretic mobility not caused by ATSK13-mediated phosphorylation

Phosphorylation can change the electrophoretic mobility of a protein, resulting in a higher (Lozano et al., 1990) or lower (Buehl et al., 2014) apparent molecular weight of the modified protein. As it was known that BAM1 can be phosphorylated by ATSK13 (Horrer, 2016), we analysed the mobility of BAM1 in *atsk13* mutants. The intensity of the lower band increased in response to stress in the mutant similarly to wild type, indicating that the shift is not due to phosphorylation by ATSK13 (Fig 3).

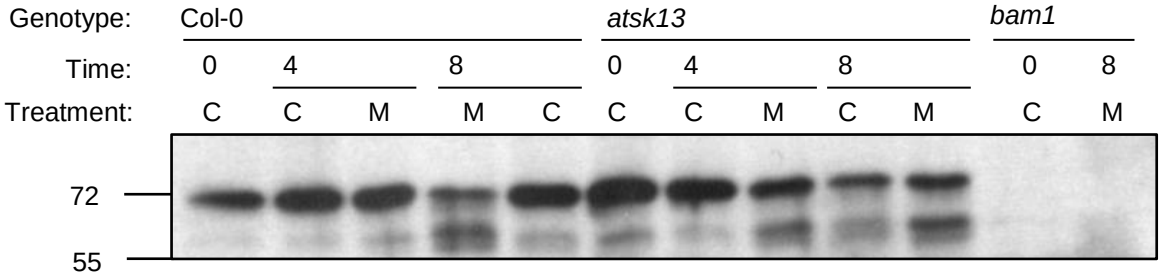


Figure 3: Immunodetection of BAM1 protein in wild-type and *atsk13* leaves after osmotic stress treatment.

The band shift is induced not only by osmotic stress alone but also depends on another unknown factor

Although the stress-induced change of electrophoretic mobility could be consistently observed during the winter of 2014/2015, it became gradually less pronounced during the spring of 2015 and was almost imperceptible by summer. We loaded new protein extracts and old extracts which showed the change in mobility on the same gel (Fig 4). The lower band was still visible in the old samples of stressed plants, demonstrating that the lack of band shift in fresh extracts was not due to any changes during the electrophoresis, blotting, or development of the pictures.

Figure 2: (A) Immunodetection of BAM1 protein in wild-type and *nced3* leaves after osmotic stress treatment. (B) BAM1 protein quantification. Densitometry analysis was used to quantify band intensities of three independent biological replicates. (C) Immunodetection of BAM1 protein in wild-type and *areb1areb2abf3* leaves after osmotic stress treatment. (D) BAM1 protein quantification. Densitometry analysis was used to quantify band intensities of three independent biological replicates

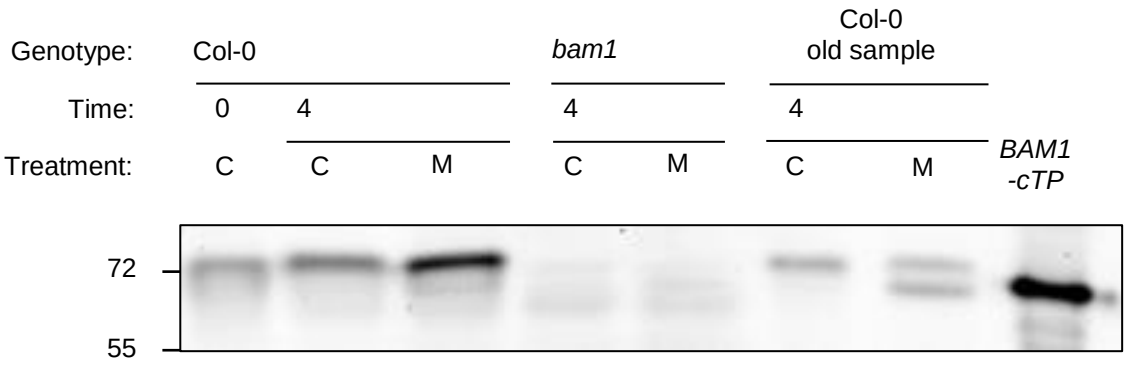


Figure 4: (A) Immunodetection of BAM1 protein in wild-type leaves of old and new samples. Recombinant BAM1 was loaded as a positive control.

Discussion

BAM1 can be modified in response to stress in an ABA-independent manner

Our results show that osmotic stress can induce post-translational modification of existing BAM1 protein, which results in a change of its electrophoretic mobility when loaded on an SDS-page. The upper band disappeared completely in the *nced3* mutant in which *BAM1* expression is no longer induced during osmotic stress (chapter1). Thus, it seems that the lower band arises from the modification of existing BAM1 protein, while the upper band in wild type extract represents newly synthesised BAM1. This explanation is consistent with the phenotype of the *areb1areb2abf3* triple mutant. In the triple mutant, stress-induced transcription is strongly reduced but not absent (chapter 1), which is reflected in the intensity of the upper band in leaf extracts. As the increase of the lower band is still apparent in both *nced3* and *areb1areb2abf3*, it is clearly independent of ABA mediated signalling.

An unknown modification leads to the change in electrophoretic mobility

Post-translational modifications of BAM1, such as formation of a disulphide bridge and phosphorylation by ATSK13 have been previously described (Sparla et al., 2006; Horrer, 2016). However, the band shift is visible under reducing conditions as well as in *atsk13* mutants, and must therefore reflect a previously unknown modification. Although the change in electrophoretic mobility is not caused by ATSK13, it could still be due to phosphorylation of BAM1 by other kinases. Alternatively, the change could also be caused by partial proteolysis which would result in smaller protein with lower molecular weight. A candidate protease is early responsive to dehydration 1 (ERD1) which is, like the modification of BAM1, strongly induced by water and salt stress but responded only slowly to ABA (Nakashima et al., 1997). To test this hypothesis we

obtained *erd1* mutant plants. Unfortunately, by the time the mutants were available, the band shift was no longer reproducible, precluding further investigation.

The band shift is only induced by specific but unknown conditions

Although the change in electrophoretic mobility was clearly visible for a period of about 6 months, efforts to reproduce it afterwards failed. As the increased intensity of the lower band can still be detected in older samples, it is not an artefact of the blotting process. Instead, it appears that there is an actual difference in the BAM1 proteins extracted at different time points. It is unclear what has caused the differential responses, but it is likely that the reconstruction work at the Institute has affected growth conditions and experiments. Relative humidity (RH) and temperature in our growth chambers were not stable and exhibited both strong diel fluctuations (~1°C and ~20% RH over the course of 24 h), as well as major long term variations (RH increased from 27% to 63% over the course of 5 months). Furthermore, new light sources and climate control aggregates were installed in our growth chamber resulting in further changes. It is likely that the variation in relative humidity affected responses to osmotic stress, and the modification of BAM1 could be observed again under extremely low relative humidity. However, recreating the exact conditions in a dedicated growth chamber was beyond the scope of this work.

Further investigation of the band shift would require the identification of the exact conditions under which the band shift is induced by osmotic stress. This would then allow a detailed analysis of the causative modification, and its role.

Alternatively, one could take advantage of the fact that the lower band is also present under control conditions, albeit at much lower intensity than the upper band. Therefore, it would be possible to isolate this species from plants using immunoprecipitation and analyse the nature of the modification. However, this approach would not reveal the physiological role of the modification.

4 – Circadian gating of stress-induced starch degradation

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Unpublished results

Synopsis: In chapter 1, we showed that high osmotic stress rapidly induces starch degradation (chapter 1). Here, we show that this induction of starch degradation is dependent on the precise timing of the day at which the stress is applied. Neither an early morning nor an evening stress induced starch degradation in WT. Preliminary analysis of mutants indicate that the lack of starch degradation in the morning can be explained by the absence of starch, as the *bam3* mutant degraded starch upon stress. In the evening, starch degradation appears to be repressed by the circadian clock and is restored in the *toc1* mutant.

Introduction

The circadian clock

Circadian clocks allow organisms to coordinate endogenous processes with the day-night cycle, and maintain periodical oscillations even in the absence of external stimuli, *e.g.* in constant light. Circadian clocks represent a widespread regulatory mechanism and are thought to have evolved several times independently (Hurley et al., 2016). As photosynthesis is only possible during the day, precise coordination of metabolic processes with the photoperiod is of particular importance for plants (Eriksson and Millar, 2003). Early research in Arabidopsis identified three transcription factors forming the core of the circadian clock: two MYB transcription factors, circadian clock associated 1 (CCA1) and late elongated hypocotyl (LHY), as well as pseudo response regulator (PRR1) which is better known as timing of CAB1 expression (TOC1). Originally it was thought that these three proteins form a negative feedback loop, resembling the circadian clock of fungi and animals. According to this model, TOC1 accumulates in the evening and activates the expression of CCA1 and LHY, which subsequently accumulate during the late night and early morning. As *TOC1* expression is repressed by CCA1 and LHY, TOC1 indirectly represses its own transcription. As TOC1 levels decline, expression of *CCA1* and *LHY* decreases, which results in a decrease of CCA1 and LHY protein, allowing *TOC1* to be expressed again, closing the circle (Alabadí et al., 2001; Eriksson and Millar, 2003).

While these three transcription factors are indeed of fundamental importance as demonstrated by the arrhythmia of the *cca1/lhy/toc1* triple mutant (Ding et al., 2007), subsequent research uncovered not only more proteins involved in the maintenance of the circadian clock, but also questioned the suggested mechanism (reviewed by Somers, 2012). According to the current model (Fig. 1), the plant circadian clock consists predominantly of repressors, with only few transcriptional activators involved. Thus, the plant clock relies on a different mechanism than the circadian clocks of other eukaryotes and resembles synthetic “repressilators” which have been constructed in bacterial cells (Elowitz and Leibler, 2000; Purcell et al., 2010). Briefly, in the morning CCA1 and LHY repress the expression of TOC1 and gigantea (GI), while promoting the expression of several other PRRs. These PRRs in turn repress the expression of CCA1 and LHY, releasing repression of TOC1 and GI. TOC1 maintains the repression of CCA1 and LHY, while GI stabilises an F-Box protein named zeitlupe (ZTL). ZTL subsequently targets TOC1 for proteasomal degradation, allowing expression of CCA1 and LHY (Greenham and Mcclung, 2015).

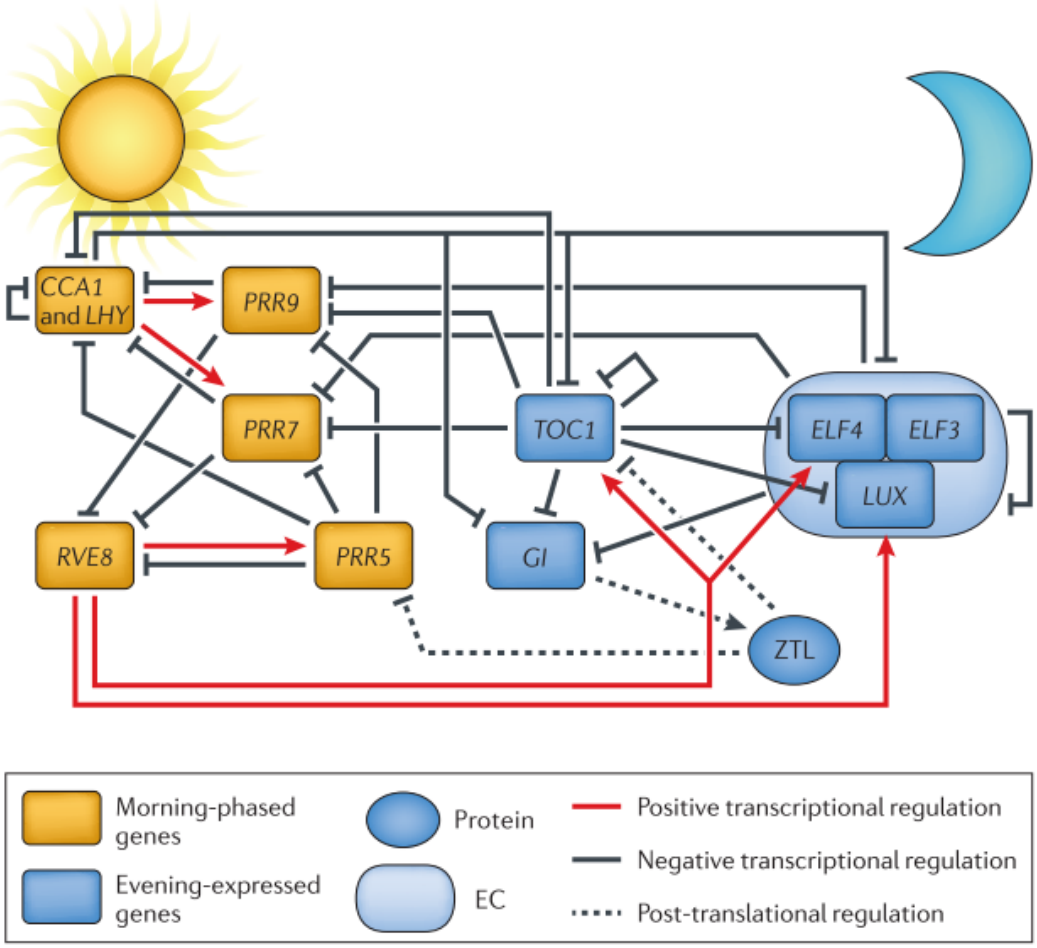

Figure 1: Model of the Arabidopsis circadian clock. Figure taken from (Greenham and Mcclung, 2015)

The circadian clock is a key regulator of gene expression, and the expression of 6% of all Arabidopsis genes show clear circadian fluctuations (Harmer et al., 2000). Through both transcriptional changes and post-translational regulation the circadian clock affects many different processes such as growth, flowering time, stomatal opening, and as described previously, also coordinates diel starch metabolism. Moreover, it has become apparent that the circadian clock also influences responses to both biotic and abiotic stresses (reviewed by Seo and Mas, 2015; Grundy et al., 2015; Greenham and Mcclung, 2015). For example, as cold stress is more likely to occur during the night, the circadian clock anticipates this possibility and the expression of c-repeat binding factors (CBF) 1, 2, and 3, which are key mediators of cold stress tolerance, peaks just before dusk (Harmer et al., 2000; Dodd et al., 2006). This process is called “circadian gating” and also affects many other stress responses (Fig. 2).

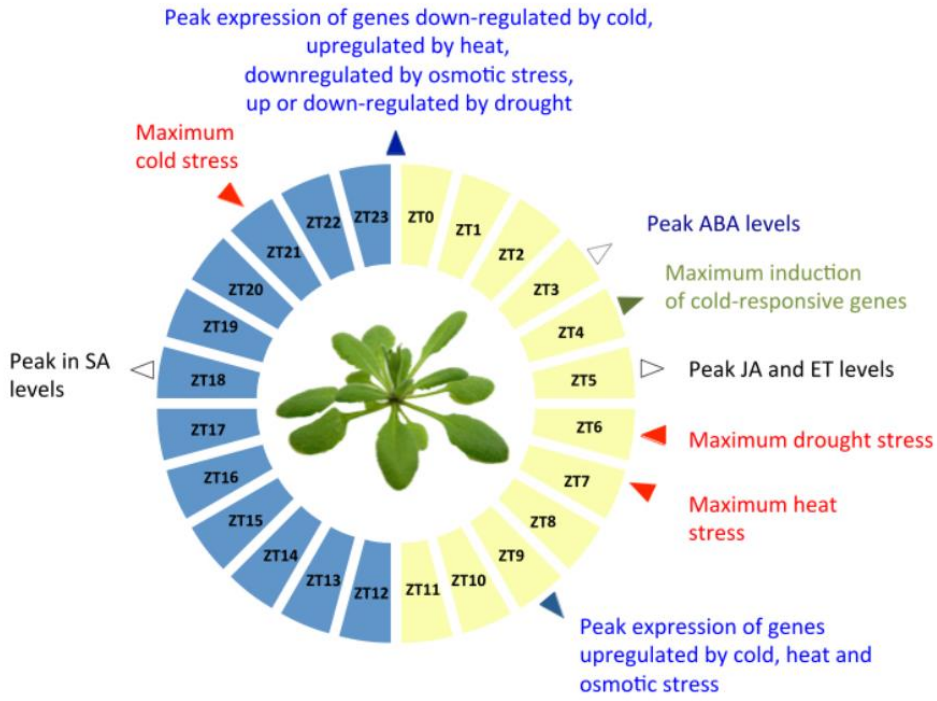

Figure 2: Timing of abiotic stress responses across the day-night cycle. The times of the day at which different types of abiotic stress are most prominent in the natural environment are indicated in red. For each type of environmental stress, the time of peak basal expression for the majority of stress-responsive genes is indicated in blue, the time of maximum accumulation of stress response hormones is shown in black, and the time of maximum responsiveness to different environmental signals is shown in green. Figure taken from (Grundy et al., 2015)

Circadian gating of ABA synthesis and signalling

ABA is a key regulator of water stress responses and both its synthesis and signalling are gated by the circadian clock (reviewed Seung et al., 2012). In Arabidopsis, endogenous ABA levels display circadian fluctuations with a peak during the early morning (Lee et al., 2006). Several ABA biosynthetic genes also show clear circadian fluctuations (Seung et al., 2012), and their expression peaks shortly before the peak of ABA content. Nonetheless, it appears that the circadian oscillation of ABA is not due to *de novo* synthesis but due to the turnover of ABA-glucose esters as the fluctuations are abolished in *bg1* plants (Lee et al., 2006). The circadian clock appears to control ABA levels through the actions of several PRRs as the *prr5prr7prr9* triple mutant no longer exhibits diel changes of ABA content, but maintains high ABA levels throughout the day (Fukushima et al., 2009).

Furthermore, the circadian clock also affects the plants ability to perceive and respond to ABA. TOC1 appears to be an important negative regulator of ABA signalling, as mutants overexpressing TOC1 are more susceptible to drought and impaired in ABA-mediated stomatal closure (Legnaioli et al., 2009). The authors speculated that TOC1

reduces ABA sensitivity by repressing the transcription of magnesium-proto-porphyrin IX chelatase subunit H (CHLH), which has been proposed as an ABA receptor (Shen et al., 2006). However, subsequent research showed that CHLH cannot bind ABA (Tsuzuki et al., 2011), questioning this theory. Nonetheless, CHLH could still be involved in ABA signalling, *e.g.* through controlling intracellular Ca^{2+} concentrations. Increased exogenous Ca^{2+} restored ABA responsiveness of stomata in CHLH deficient plants (Tsuzuki et al., 2011). Moreover, TOC1 was shown to bind the promoters of many genes involved in the canonical ABA signalling pathways (Huang et al., 2012; Grundy et al., 2015), indicating that TOC1 could control ABA transduction independently of CHLH. The role of TOC1 in ABA signalling was also corroborated using computational models, which accurately simulated the changes observed *in vivo* (Pokhilko et al., 2013). Thus, the function of TOC1 as a repressor of ABA signalling is well established even if the precise mechanism is still unclear.

In addition to affecting ABA content and signalling, the circadian clock also directly affects expression of ABA responsive genes, and over 40% of ABA responsive genes were found to be under circadian control (Covington et al., 2008). Binding sites for CCA1 and LHY were found to be common in ABA responsive genes, indicating that their expression can be directly affected by the circadian clock (Huang et al., 2007).

Due to the known influence of the circadian clock on both ABA signalling and diel starch metabolism we wanted to investigate if stress-induced starch degradation is also affected by the time of day at which the stress is imposed.

Material and Methods

Plant growth and osmotic stress induction

Plants were grown in hydroponics as described in chapter 1. Osmotic stress was induced as described in chapter 1 by adding either a 300mM mannitol solution. The following genotypes were used:

Allele	Line	Publication
bam1	SALK_039895	(Sparla et al., 2006)
bam3	CS92461	(Fulton et al., 2008)
toc1-101	-	(Kaczorowski, 2004)

Quantification of starch

Starch was extracted and quantified using glucose-6-phosphate dehydrogenase assay as described in chapter 1.

Results

Stress-induced starch degradation in the early morning occurs in bam3 *mutant but not in wild type*

We exposed plants to osmotic stress at the end of the night rather than after three hours of illumination as it was done in chapter 1. As expected, both wild-type Col-0 and *bam1* mutant plants contained no starch at this time point, while *bam3* mutant plants still contained starch (Fig 3) (Fulton et al., 2008). All three genotypes synthesised starch over the course of the experiment under control conditions. However, in response to osmotic stress, only the *bam3* mutant, which did not exhaust its starch reserves during the night, degraded starch. In contrast, starch content of Col-0 and *bam1* mutants was unchanged by osmotic stress at the beginning of the day.

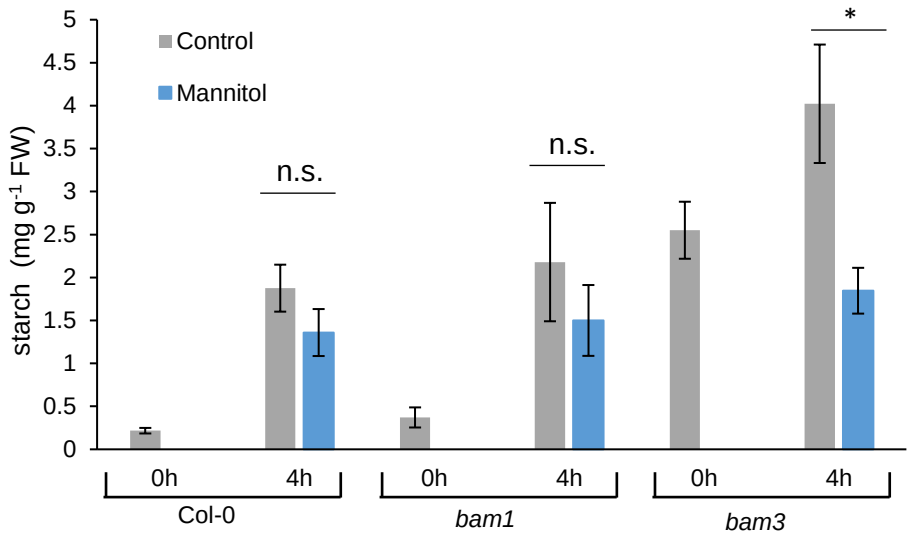

Figure 3: Leaf starch content of plants exposed to osmotic stress at the end of the night. Values are means ± SE (n = 10). FW, fresh weight.

Stress-induced starch degradation in the late afternoon occurs in the toc1 mutant but not in wild type

We also exposed plants to osmotic stress after 8 hours of illumination. Although both Col-0 and *bam1* accumulated starch at the beginning of the stress, osmotic stress failed to induce starch degradation in either genotype (Fig 4A). Interestingly, starch degradation was induced in response to an unexpectedly early night, showing that plants have the capacity to degrade starch at this time of the day.

As ABA is required for stress-induced starch degradation, and TOC1 has been proposed to repress ABA signalling through increased expression towards the end of the day, we tested whether osmotic stress induced starch degradation in *toc1* mutants. Preliminary results indicate that this is indeed the case, and that in *toc1*

mutants osmotic stress induces starch degradation both at noon and in the evening (Fig 4B).

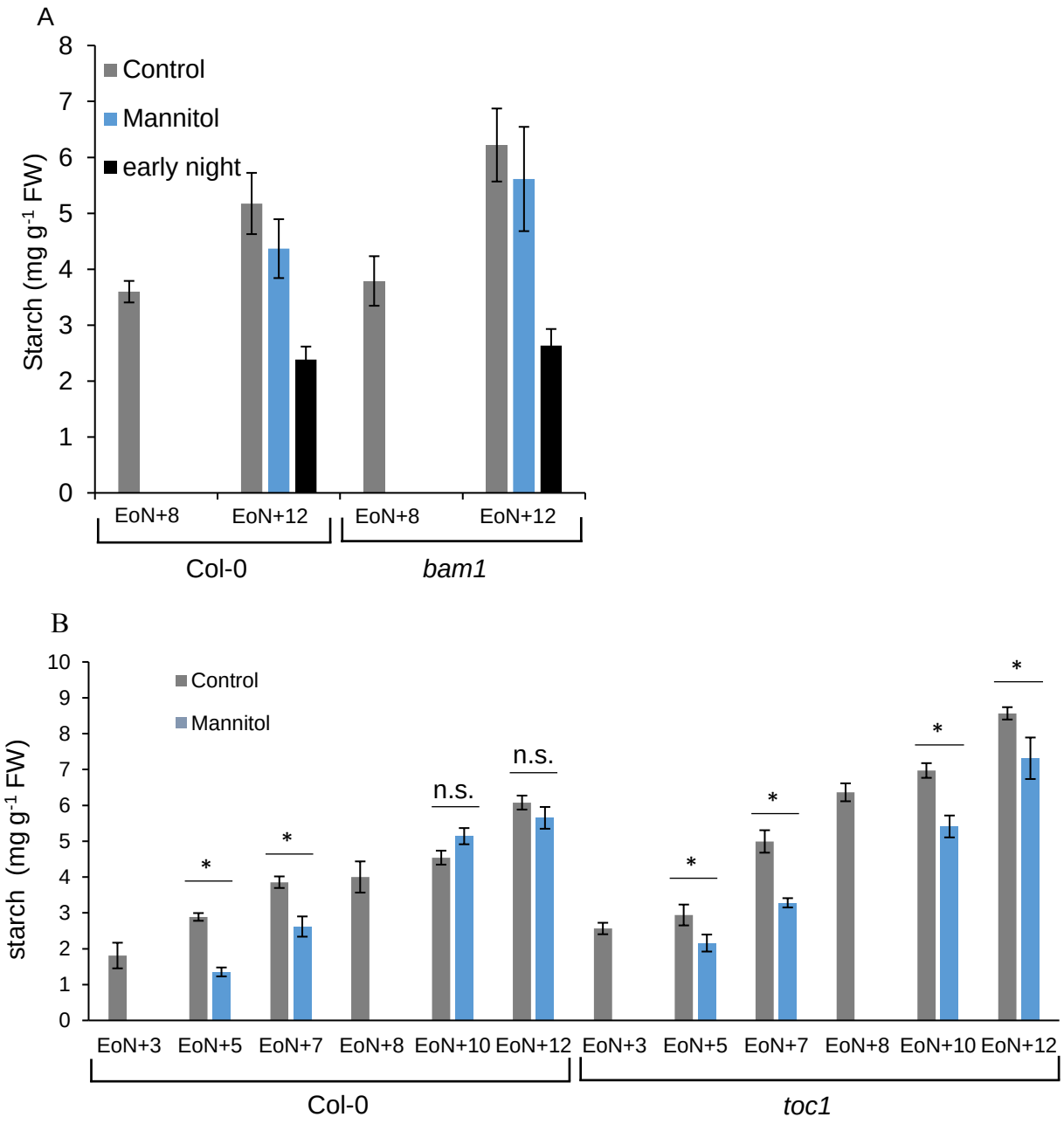

Figure 4: (A) Leaf starch content of plants exposed to osmotic stress exposed to unexpectedly early night after 8 hours of illumination. (B) Leaf starch content of plants exposed to osmotic stress at EoN+3 or EoN+8. Values are means ± SE (n = 5). FW, fresh weight.

Discussion

Starch degradation is only induced by stress if sufficient starch reserves are present

While osmotic stress induced starch degradation in Col-0 after three hours of illumination, no starch degradation was induced at the beginning of the day when the starch reserves of Col-0 are depleted. Interestingly, starch degradation was induced in *bam3* mutants which did not fully degrade starch during the night (Fig 3). This suggests that starch degradation is only induced by osmotic stress if sufficient starch is present.

Previous investigations of diel starch turnover indicated that plants can assess their starch reserves and adjust the rate of starch degradation accordingly (Scialdone et al., 2013). This adjustment ensures that starch reserves are full consumed by the end of the night but not before, allowing the plant to efficiently use stored carbon. Our results suggest that stress-induced starch degradation is controlled in a similar manner.

To corroborate this hypothesis it will be necessary to test the response to osmotic stress of other mutants with a starch excess phenotype comparable to *bam3*. Potential candidates are *bam4*, *dpe1*, and *lsf1*. If the stress-induced starch degradation in the morning indeed occurs when starch is present, osmotic stress should induce starch degradation in all of these mutants. It was previously shown that the rate of starch degradation can no longer be adjusted in *pwd* mutants (Scialdone et al., 2013) in response to unexpectedly early night. Thus, analysing the response of *pwd* mutant plants to osmotic stress in the morning could reveal if PWD is also required to adjust the rate of starch degradation under these conditions.

To identify the mechanism preventing starch degradation in the morning it would be necessary to quantify the expression of *BAM1* and *AMY3* in response to stress. If the repression of starch degradation is achieved by transcriptional regulation, *BAM1* and *AMY3* should not be induced by osmotic stress in Col-0 plants but should be induced in the leaves of the starch excess mutants. Conversely, if both genes are induced by stress in Col-0 this would indicate that starch degradation is repressed by post-translational regulation.

Towards the end of the photoperiod stress-induced starch degradation is supressed in an TOC1-dependent manner

Curiously, starch degradation was also not induced when the stress was applied towards the end of the day (8h of light), even though at this time plants accumulated large amounts of starch. As starch degradation was induced by unexpectedly early nightfall, we reason that plants are in principle able to induce starch degradation at this

time of the day, and that the effect is specific to stress-induced starch degradation. As stress-induced starch degradation was restored in *toc1* mutants, its repression appears to be regulated by the circadian clock. It was previously shown that TOC1 can repress ABA-mediated stomatal closure (Legnaioli et al., 2009), and as ABA is required for stress-induced starch degradation (chapter 1), it is likely that TOC1 prevents starch degradation through its effect on ABA signalling.

To test this hypothesis it will be necessary to investigate the expression of *BAM1* and *AMY3* in response to ABA and osmotic stress in the evening, in both Col-0 and *toc1* plants. If the suppression of stress-induced starch degradation is indeed mediated through transcriptional repression of starch degrading enzymes it would be expected that *AMY3* and *BAM1* expression is reduced in Col-0 but restored in *toc1*. To analyse whether this represents a general repression of ABA-induced gene expression, the expression of *RD29A,* a marker for ABA-induced gene expression, should also be analysed under these conditions.

In our preliminary experiments, we observed that *toc1* mutant plants accumulated slightly more starch than Col-0 plants over the course of the day. It was shown that mutations in other clock components affect the diel starch turnover (Graf et al., 2010). However, so far there have been no reports of changed starch metabolism in *toc1* mutants. Therefore, the apparent increase in starch synthesis in *toc1* mutants should be investigate further.

It would be also interesting to investigate the behaviour of TOC1-overexpressing plants (TOC1OX) under osmotic stress. Previous studies showed that TOC1OX plants were impaired in ABA mediated stomatal closure and more susceptible to drought (Legnaioli et al., 2009). Thus, it is likely that stress-induced starch degradation is impaired in TOC1OX plants through the day.

Lastly, the dependence of stress-induced starch degradation on the timing of the stress raises the possibility that responses to stress could depend on the photoperiod the plants are grown in. All experiments were conducted on plants grown in 12 h light/12 h dark cycles, and it would be interesting to investigate stress responses of plants grown in short day (8h light/16 hours darkness) or long day (16 hours light/8 h darkness) or even under continues light.

5 – Starch as a determinant of plant fitness under abiotic stress

Matthias Thalmann, Diana Santelia

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Synopsis: We surveyed 36 studies about the impact of abiotic stress on carbohydrate metabolism. The majority of these (23) reported a decrease of starch content during stress, in particular during drought, heat, and osmotic stress. The investigated organisms included economically important crop plants, but also basal land plants and algae, indicating that starch degradation is a conserved response to these stresses. Interestingly, a minority of the studies found increased starch content during stress. As these studies were done mostly on plants exposed to cold or salt stress, it appears that different stresses may elicit different responses.

New Phytologist

Review

Tansley insight

Starch as a determinant of plant fitness under abiotic stress

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Key words: abiotic stress, abscisic acid (ABA), alpha-amylase, beta-amylase, starch, sugars.

Summary

Abiotic stresses, such as drought, high salinity and extreme temperatures, pose one of the most important constraints to plant growth and productivity in many regions of the world. A number of investigations have shown that plants, including several important crops, remobilize their starch reserve to release energy, sugars and derived metabolites to help mitigate the stress. This is an essential process for plant fitness with important implications for plant productivity under challenging environmental conditions. In this Tansley insight, we evaluate the current literature on starch metabolism in response to abiotic stresses, and discuss the key enzymes involved and how they are regulated.

I. Introduction

Starch is the major carbohydrate storage in plants. It is a simple molecule composed of glucose residues which are linked to each other by α-1,4-linkages with occasional α-1,6-branches, forming osmotically inert, semi-crystalline and dense granules (Zeeman *et al.*, 2010). Starch is most commonly associated with storage organs such as roots, rhizomes, tubers, stems and seeds, which represent not only the major source of carbohydrates in the human diet, but also provide raw material for industrial purposes (Santelia & Zeeman, 2011). However, the view of starch as merely an inert long-term storage molecule has been increasingly challenged, and a surprising plasticity of starch metabolism has been discovered as it turned out to be intimately integrated with plant biology. In storage organs, starch is synthesized from transported sucrose and can be stored over the seasons, or even many years, fueling regrowth or seedling establishment at the start of the growing season. In photosynthetic cells, starch is synthesized mostly using a fraction of the CO_2-fixed carbon and temporally stored in the chloroplasts. This starch is called 'transitory' as it is synthesized and degraded within a 24-h window. However, synthesis and degradation may occur at different times of day, and starch may serve different functions according to the cell type from which it is derived and the external environmental conditions. In this review, we focus on the metabolism of transitory starch in response to abiotic stresses, addressing how reorganization of starch metabolism contributes to plant fitness and survival under challenging growth conditions. Furthermore, we present the enzymes known so far to be involved in

the stress-induced starch degradation and how they are regulated.

II. Multiple functions of starch metabolism

Starch metabolism is best understood in leaves, where starch is synthesized during the day and mobilized during the following night to guarantee a steady supply of carbon and energy when photosynthesis is not possible. Adjustments of starch degradation rate occur under changing photoperiods or in response to a sudden early or late dusk to ensure optimal carbon supply for continued growth during the night (Stitt & Zeeman, 2012). In the model plant *Arabidopsis thaliana*, starch is degraded via an intricate network of reactions involving the synergistic action of multiple enzymes (reviewed by Streb & Zeeman, 2012). The main hydrolytic enzyme during night-time starch degradation in the leaf chloroplasts is β-amylase (mainly BAM3), which attacks the nonreducing end of starch to release maltose (Fig. 1a; Fulton *et al.*, 2008). BAM3 works synergistically with debranching enzymes (mainly the isoamylase ISA3), which are responsible for the hydrolysis of the α-1,6-branches of starch into short soluble malto-oligosaccharides. The activity of BAM3 and ISA3 depends on the reversible phosphorylation of the starch granule surface by the glucan water dikinase (GWD) and the phosphoglucan water dikinase (PWD), and the phosphoglucan phosphatase starch excess 4 (SEX4; Fig. 1a; Edner *et al.*, 2007; Kötting *et al.*, 2009). Starch phosphorylation increases the hydration status of the granule–stroma interface, disrupting its crystallinity and thereby facilitating the actions of the glucan-hydrolyzing enzymes.

Interestingly, the Arabidopsis genome encodes additional starch-related enzymes, such as phosphorylases, α-amylases and limit dextrinase, which are able to degrade starch but seem not to be required for leaf starch turnover under standard growth conditions (Zeeman *et al.*, 2004; Yu *et al.*, 2005; Streb *et al.*, 2012).

Besides its well-described function in supporting night-time metabolism, starch plays other fundamental roles in plant physiology. For example, in the guard cells which border the stomatal pores that control water and CO_2 exchange with the environment, starch is degraded very rapidly upon light exposure, helping to generate organic acids and sugars to increase guard cell turgor and promote stomatal opening (Horrer *et al.*, 2016). In this cell type, the major starch degrading enzyme is BAM1, which works synergistically with the α-amylase 3 (AMY3) to efficiently degrade starch (Valerio *et al.*, 2011; Horrer *et al.*, 2016). Loss of BAM1 and AMY3 alone or in combination does not affect starch metabolism in mesophyll cells under normal conditions (Horrer *et al.*, 2016; Thalmann *et al.*, 2016), indicating that these are specialized enzymes which have tissue-specific functions.

Starch is present in small amounts also in the amyloplasts of the root-cap columella cells, where it is necessary for full gravity perception and sensitivity (Weise & Kiss, 1999). However, the glucan enzymes operating in this cell type and the underpinning molecular mechanisms are mostly unknown. In reproductive tissues, starch turnover during flower, early embryo and silique development is functionally linked to cell division and differentiation rather than to storage functions (Andriotis *et al.*, 2010; Hedhly *et al.*, 2016). The glucose-6-phosphate (Glc6P)/phosphate antiporter GPT1 was recently identified as the putative translocator of Glc6P for starch biosynthesis in reproductive tissues (Hedhly

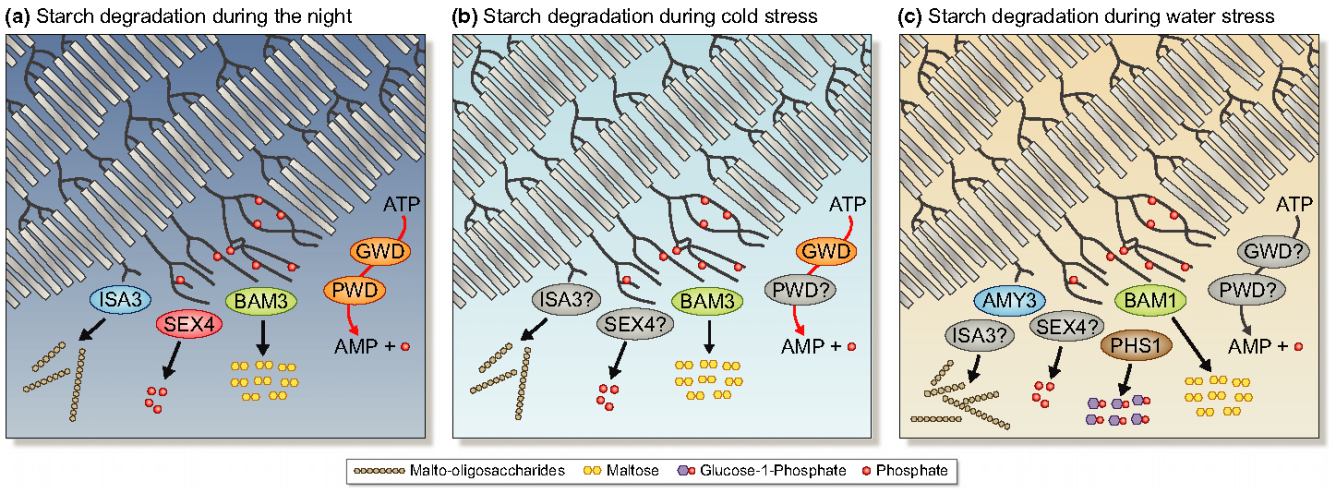

Fig. 1 Pathways of starch degradation under different conditions. (a) During the night, glucan water dikinase (GWD) and phosphoglucan water dikinase (PWD) phosphorylate the starch granule surface, disrupting its semi-crystalline structure. Subsequently, BAM3 attacks the exposed ends of the glucan chains releasing maltose. As β-amylase (BAM) cannot degrade past a phosphorylated glucose unit nor hydrolyze glucosidic bonds which are too close to a branch point, the activity of glucan phosphatase SEX4 and the debranching enzyme ISA3 are required to accomplish complete starch degradation. (b) GWD and BAM3 are also involved in starch degradation in response to cold stress, suggesting that a similar starch degradation pathway may operate under these conditions. However, the involvement of the other night-time starch-degrading enzymes needs experimental verification, and potential differences cannot be ruled out at this stage. (c) By contrast, starch degradation induced by water deficit or osmotic stress depends on a set of enzymes which are not required for night-time starch metabolism. These are mainly BAM1 and AMY3, which release (respectively) maltose and soluble linear and branched malto-oligosaccharides. The starch phosphorylase PHS1 also contributes to starch degradation under drought stress by releasing glucose-1-phosphate from the nonreducing ends of the glucan chains. If, or which, other starch-degrading enzymes are potentially involved in this process is not known.

et al., 2016). As mesophyll cells cannot import Glc6P efficiently, this again shows that starch turnover in different cell-types requires the activity of specialized enzymes, explaining why so many starch-related enzymes exist, with some involved in night-time metabolism in the leaves and other required for starch metabolism in other plant cell types.

III. Starch metabolism during abiotic stress

Starch is also emerging as a key molecule in mediating plant responses to abiotic stresses, such as water deficit, high salinity or extreme temperatures. Under these challenging environmental conditions, plants generally remobilize starch to provide energy and carbon at times when photosynthesis may be potentially limited. The released sugars and other derived metabolites support plant growth under stress, and function as osmoprotectants and compatible solutes to mitigate the negative effect of the stress (Krasensky & Jonak, 2012). Sugars also can act as signaling molecules, which cross-talk with the ABA-dependent signaling pathway to activate downstream components in the stress response cascade (Rook *et al.*, 2006).

We conducted an extensive survey of the current literature on starch metabolism under abiotic stresses. In the majority (23 of 36) of the considered studies, leaf starch content was reported to decrease in response to abiotic stress, independently of the analyzed species (Table 1). For example, in the green alga *Dunaliella tertiolecta*, starch degradation was seen in response to salt stress (Goyal, 2007); the desiccated shoots of the moss *Polytrichum formosum* were devoid of starch (Pressel *et al.*, 2006); and starch was degraded upon stress also in woody plants such as the lychee tree (*Litchi chinensis*) and herbaceous species such as barley (*Hordeum vulgare*) (Villadsen *et al.*, 2005; Damour *et al.*, 2008). Degradation of starch in response to stress often has been correlated with improved tolerance. In the moss *Physcomitrella patens*, ABA-induced starch degradation resulted in increased freezing tolerance (Nagao *et al.*, 2005). González-Cruz & Pastenes (2012) found that a drought-resistant variety of broad bean (*Phaseolus vulgaris*) degraded more starch than a drought-sensitive variety. Likewise, Cuellar-Ortiz *et al.* (2008) reported an increased accumulation of carbohydrates only in the pods of a drought resistant variety of soybean (*Glycine max*).

That said, several studies also reported an increase in starch accumulation under stress. Examples include the halophyte *Thellungiella halophila* (Wang *et al.*, 2013), the green alga *Chlamydomonas reinhardtii* (Siaut *et al.*, 2011), and in some cases also Arabidopsis (Kaplan & Guy, 2004; Skirycz *et al.*, 2010). The reason for this discrepancy is unclear. Different stresses may induce different responses: indeed, increased starch content was mainly seen in response to high salinity or cold stress (see Table 1); the response might depend on the exact experimental conditions; and halophytes may have evolved specific responses to high salinity, for example by using starch to scavenge sodium (Kanai *et al.*, 2007). A puzzling example is cold-stress treatment, which resulted in increased starch content, along with the accumulation of the major starch-degrading product maltose (Kaplan & Guy, 2004).

These different, sometime opposing, rearrangements of starch metabolism upon stress highlight the plasticity of starch and demonstrate that it cannot be merely considered as a storage compound. Starch plasticity also becomes evident when comparing the stress response of various plant tissues. Cuellar-Ortiz *et al.* (2008) reported that starch was depleted in broad bean leaves but accumulated in pods in response to drought stress. In broad bean and tomato (*Solanum lycopersicum*), drought and salt stress, respectively, led to the accumulation of starch in fruits (Cuellar-Ortiz *et al.*, 2008; Yin *et al.*, 2010), suggesting that starch may play a different role in fruits and leaves under stress. However, in both tissues, soluble sugars accumulated in high amounts (Cuellar-Ortiz *et al.*, 2008; Yin *et al.*, 2010). Notable differences in stress-induced reorganization of starch metabolism also were observed within different vegetative tissues. In drought-stressed plants of white lupin (*Lupinus albus*), soluble sugars dropped in the leaf blade, but accumulated in the petiole (Pinheiro *et al.*, 2001). In the common reed (*Phragmites australis*), starch content increased in all vegetative tissues but the amount at the base of the shoot was more than two-fold higher than that in the upper part of the shoot (Kanai *et al.*, 2007).

Altogether, these studies demonstrate that soluble sugars unequivocally accumulate in response to stress, and that the type of sugar depends on the plant species and the stress treatment (Table 1). In many cases, maltose also accumulates, suggesting that the activation of starch degradation under stress is a common plant response and does contribute to sugar accumulation (Kaplan & Guy, 2004; Yano *et al.*, 2005; Thalmann *et al.*, 2016). However, sugars may also derive from photosynthetic carbon assimilation or accumulate because of decreased demand, as a consequence of growth limitation.

IV. Enzymes involved in stress-induced reorganization of starch metabolism

Work done with Arabidopsis mutants revealed that starch degradation under stress does not necessarily involve the hydrolytic enzymes of the night-time leaf starch degradation. It rather seems that different combinations of starch-degrading enzymes operate under different stresses. GWD and BAM3 are the major starch-degrading enzymes under cold stress (Fig. 1b). Mutants deficient in GWD showed reduced sugar accumulation during cold acclimation, increased electrolyte leakage, and reduced survival rate during following exposure to freezing temperatures (Yano *et al.*, 2005). *bam3* mutants accumulated lower amounts of sugars and had impaired photosynthesis during cold stress (Kaplan & Guy, 2005). Thus, starch degradation under cold stress seems to share some similarities with starch degradation at night. However, the function of many other important night-time starch degrading enzymes during cold stress remains to be investigated, making it difficult to draw conclusions at this stage.

Under water deficit or osmotic stress, *bam3* mutants, although compromised in normal night-time starch metabolism, could efficiently activate starch degradation (Monroe *et al.*, 2014; Thalmann *et al.*, 2016; Zanella *et al.*, 2016). By contrast, Arabidopsis mutants deficient in BAM1, AMY3 or both, had severe

Table 1 Impact of different stress treatments on starch and soluble sugar metabolism in different plant species

Plant species	Stress	Changes in carbohydrates	Changes in gene expression or enzyme activity	Reference
Polytrichum formosum	Desiccation (7.5% water content)	Starch ↓	nd	Pressel *et al.* (2006)
Solanum tuberosum ssp. *andigena*	Drought (withholding water for one or two cycles until net assimilation was below 1 μm CO_2 m^{-2}, followed by rewatering)	nd	Starch synthases ↓ SPS ↑ AGPase ↓ β-amylase ↑	Watkinson *et al.* (2008)
Triticale AC Certa Triticale AC Ultima Triticale Blue Alta	Drought (reduction of soil moisture to 55–60%, 30–35%, and 10–15%)	Starch ↓	AGPase ↓ Starch synthases ↓ Starch Branching enzymes ↓	He *et al.* (2012)
Arabidopsis thaliana Col-0	Drought (reduction of soil water content to 0.25 g water/dry weight and 0.18 g water/dry weight)	Hexoses ↑ Suc ↑ Starch ↓	AGPase ↑ Invertase ↓	Hummel *et al.* (2010)
Phaseolus vulgaris L. Canario 60 *Phaseolus vulgaris* L. Nueva Granada	Drought (water content reduced to 60% or 30% of field capacity)	*In resistant cultivar only*: Glc in pods ↑ Fru in pods ↑ Suc in pods ↑ Starch in pods ↑ In both cultivars: Starch in leaves ↓	Resistant only: Sucrose synthase ↑ Neutral invertase ↓	Cuellar-Ortiz *et al.* (2008)
Leymus chinensis	Drought (water content reduced to five different amounts) High temperature during day and night (30°C/25°C) and combination of the two	Starch ↓	nd	Xu *et al.* (2009)
Oryza sativa L. japonica Wuyujing 3 *Oryza sativa* L. indica Yangdao 4	Drought (water potential reduced to −0.05 MPa)	Soluble sugars ↑ Suc ↑ Starch ↓	α-amylase ↑ β-amylase ↑ SPS ↑	Yang *et al.* (2001)
Glycine max L. Merr.	Drought (water withheld for 21 d)	*In leaves and pods:* Hexose ↑ *In leaves only:* Suc ↓ Starch ↓	Soluble invertase in pods ↓	Liu *et al.* (2004)
Litchi chinensis cv Kawaï Mi	Drought (withhold water for 5.5 months)	Soluble sugars ↑ Starch ↓	nd	Damour *et al.* (2008)
Phaseolus vulgaris L. Arroz Tuscola *Phaseolus vulgaris* L. Orfeo INIA	Drought (watering reduced to 1/6 of control)	Starch ↓	nd	González-Cruz & Pastenes (2012)
Lupinus albus L. cv Rioi Maior	Drought (withhold water for 13 d)	Glc ↑ Fru↑ Suc ↑	Sucrose synthase ↑ Acidic invertase ↑	Pinheiro *et al.* (2001)
Hordeum vulgare L. cv Bonus	Drought (withhold water for 3 d) Osmotic stress (1 M sorbitol)	Glc ↑ Fru↑ Fru-2,6-Bisphosphate ↑ Suc ↓ Starch ↓	F6p2K:F-2,6-BPase ratio ↑ PFP ↓ SPS ↓ AGPase ↓ Invertase ↓	Villadsen *et al.* (2005)
Arabidopsis thaliana Col-0	Drougth (reduction of soil water content to 30%, or complete withhold of water)	Starch ↓	α-amylase ↑ β-amylase ↑	Harb *et al.* (2010)

Table 1 (Continued)

Plant species	Stress	Changes in carbohydrates	Changes in gene expression or enzyme activity	Reference
Trifolium repens L. cv Huria	Drougth (watering reduced to 10% of control)	Glc ↑ Suc ↑ Soluble sugars ↑ Starch ↓	nd	Lee *et al.* (2008)
Arabidopsis thaliana Col-0	Freezing (−3°C to −15°C) with or without acclimation at 2°C	Glc ↑ Fru ↑ Maltose ↑ Suc ↑ Starch ↓	GWD ↑	Yano *et al.* (2005)
Arabidopsis thaliana Col-0 *Arabidopsis thaliana* Ler	High temperature (30°C)	Soluble sugars ↓ Starch ↓	nd	Vasseur *et al.* (2011)
Physcomitrella patens	Low temperature (−16°C to 4°C) ABA treatment (0.1–100 mM)	Starch ↓ soluble sugars ↑	nd	Nagao *et al.* (2005)
Solanum tuberosum L. cv Desirée *Solanum tuberosum* L. cv Russet Burbank	Low temperature (2°C)	Reducing sugars ↑	α-amylase ↑ β-amylase ↑ Phosphorylase ↑	Sitnicka & Orzechowski (2014)
Arabidopsis thaliana Col-0	Low temperature (4°C)	Glc ↑ Fru↑ Suc ↑ Maltose ↑ Starch ↑	BAM3 ↑	Kaplan & Guy (2005)
Arabidopsis thaliana Col-0	Low temperature (4°C)	Glc ↑ Fru↑ Suc ↑ Starch ↑	nd	Hoermiller *et al.* (2016)
Arabidopsis thaliana Col-0	Low temperature (4°C) High temperature (40°C)	During cold stress: Starch ↑ Maltose ↑ Glc ↑ Fru ↑ Suc ↑ Trehalose ↑ During heat stress: Maltose ↑ Starch ↓	BAM3 cold ↑ BAM1 heat ↑	Kaplan & Guy (2004)
Arabidopsis thaliana Col-0	Low temperature (6°C)	Soluble sugars ↑ Starch ↓	β-amylase ↑	Sicher (2011)
Solanum tuberosum L. cv Desirée	Osmotic stress (100, 200, 300, 500 mM mannitol)	Suc ↑ Starch ↓	Starch synthesis ↓ sucrose synthesis ↑ SPS ↑	Geigenberger *et al.* (1997)
Arabidopsis thaliana Col-0	Osmotic stress (150 mM mannitol)	Maltose ↑ Suc ↑ Starch ↑	nd	Zanella *et al.* (2016)
Arabidopsis thaliana Col-0	Osmotic stress (200 mM mannitol) Cold stress (4°C)	Reducing sugars ↑ Starch ↑	Osmotic: BAM1 ↑ cold: BAM3 ↑	Monroe *et al.* (2014)
Arabidopsis thaliana Col-0	Osmotic stress (25 mM mannitol)	Soluble sugars ↑ Starch ↑	nd	Skirycz *et al.* (2010)

Table 1 (Continued)

Plant species	Stress	Changes in carbohydrates	Changes in gene expression or enzyme activity	Reference
Arabidopsis thaliana Col-0	Osmotic stress (300 mM mannitol) ABA treatment (100 mM)	Glc ↑ Fru ↑ Maltose ↑ Suc ↑ Starch ↓	BAM1 ↑ AMY3 ↑	Thalmann *et al.* (2016)
Arabidopsis thaliana Col-0	Osmotic stress (450 mM mannitol)	Starch ↓	BAM1 ↑	Valerio *et al.* (2011)
Arabidopsis thaliana Col-0	Oxidative stress (50 μM methyl viologen)	Suc ↓ Starch ↓	Starch synthesis ↓ Sucrose synthesis ↓ Starch degradation ↑	Scarpeci & Valle (2008)
Arabidopsis thaliana Col-0	Salt stress (150 mM) ABA treatment (25 mM)	Suc ↑ Maltose ↑ Starch ↓	AGPase ↑ Starch synthase ↑ BAM1 ↑	Kempa *et al.* (2008)
Phragmites australis	Salt stress (100 mM)	Starch ↑	nd	Kanai *et al.* (2007)
Solanum lycopersicum L. cv Micto-Tom	Salt stress (160 mM)	In Fruits: Glc ↑ Fru↑ Starch ↑	nd	Yin *et al.* (2010)
Dunaliella tertiolecta	Salt stress (170 or 700 mM)	Glycerol ↑ Starch ↓	nd	Goyal (2007)
Oryza sativa L. cv Tainung 67	Salt stress (200 mM NaCl) Osmotic stress (20% (w/v) PEG6000)	Starch ↓	GBSS ↓ AGPase ↓	Chen *et al.* (2008)
Thellungiella halophila *Shandong ecotype*	Salt stress (200 mM, 400 mM, 600 mM NaCl)	Soluble sugars ↑ Starch ↑	AGPase ↑ Starch synthases ↑	Wang *et al.* (2013)
Chlamydomonas reinhardtii *(multiple strains)*	Salt stress (25, 50, 100 mM)	Starch ↑	nd	Siaut *et al.* (2011)

SPS, Sucrose-phosphate synthase; AGPase, ADP-Glucose pyrophosphorylase; GBSS, Granule bound starch synthase; PFP, diphosphate-frucotse-6-phosphate 1-phosphotransferase; F6p2K, Fructose-6-phosphate 2-kinase; F-2,6-BPase, Fructose-2,6-bisphosphate phosphatase; Glc, Glucose; Fru, Fructose; Suc, Sucrose; nd, not determined.

reduction in osmotic stress-induced starch degradation, accumulated less sugars and proline, and showed a reduced water uptake ability (Thalmann *et al.*, 2016; Zanella *et al.*, 2016). Plants lacking the α-glucan phosphorylase (PHS1) were also reported to be more sensitive to drought and high salinity (Zeeman *et al.*, 2004). Starch degradation induced by water deficit or osmotic stress seems, therefore, to be mediated by BAM1, AMY3 and PHS1, which are all hydrolytic enzymes not required for night-time starch metabolism (Fig. 1c). This is similar to guard cells, although the function of PHS1 in this cell type has not been investigated.

These contrasting phenotypes hint at an intricate network of differential enzyme regulation, leading to isoform subfunctionalization, plasticity of starch turnover and, ultimately, increased fitness (Box 1).

V. Regulation of starch metabolism during abiotic stress

Our current knowledge on the mechanisms controlling starch metabolism is still in its infancy. Despite being well documented that both light intensity and the circadian clock are essential regulatory factors (Graf *et al.*, 2010), how they ultimately impact on the activity of the starch enzymes is not fully understood. Transcript levels for many of the enzymes of starch degradation in the Arabidopsis leaf at night co-ordinately peak towards the end of the day and fall to low amounts at the end of the night. However, this gene expression pattern often does not correlate with the amount of the corresponding protein or the enzyme activity (Smith *et al.*, 2004).

Transcriptional regulation appears to play a role in the regulation of starch metabolism in response to stress. Several transcriptomic studies have reported ABA-dependent induction of a multitude of genes involved in primary carbohydrate metabolism (Zeller *et al.*, 2009; Choudhury & Lahiri, 2011; Yoshida *et al.*, 2014). The rapid biosynthesis of ABA is recognized as one of the pivotal events in abiotic stress tolerance, and it was correlated with stress-induced alterations in starch metabolism (Kempa *et al.*, 2008). Exogenous ABA induced starch degradation in *P. patens*, leading to increased freezing tolerance (Nagao *et al.*, 2005). Very recently, Thalmann *et al.* (2016) showed that, in Arabidopsis, exogenous ABA induced the expression of *BAM1* and *AMY3*, which was accompanied by increased BAM1 enzyme activity and induction of starch degradation. Molecular genetic analyses further demonstrated that the

Box 1 What is plant fitness?

Although we often think of fitness as the ability of an individual to withstand adverse conditions, from an evolutionary point of view, fitness is defined as an individual's ability to propagate its genes through its offspring. In the case of plants, maternal fitness depends on the number of vital seeds an individual successfully produces under adverse environmental growth conditions. A major effect of abiotic stresses is seed abortion, which reduces the number of offspring, but also the yield in many important crop species. Seed abortion is caused by reduced photosynthesis which limits the carbon supply and also results in reduced biomass production. Remobilization of starch under stress provides an alternative source of energy and carbon, which promotes seeds to mature into healthy offspring even under stressful environmental conditions. Plants also use the released carbon for the biosynthesis of compatible solutes, allowing the plant to mitigate the effects of stress, and therefore increasing the chances of a successful reproduction (Fig. 2).

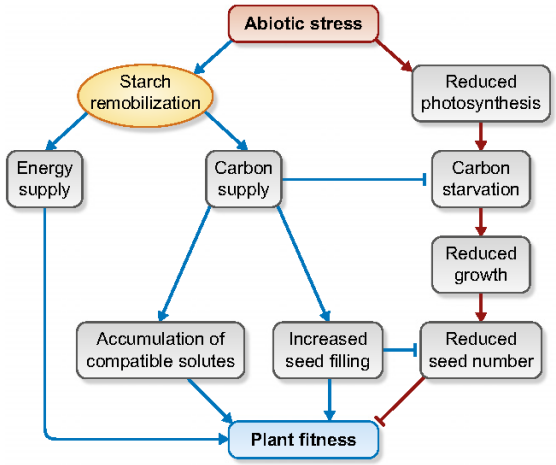

Fig. 2 Starch remobilization during abiotic stress improves plant fitness.

ABA effect on starch metabolism is partly mediated by the canonical ABA signaling pathway (reviewed by Fujita *et al.*, 2013), involving the SNF1-related kinase 2s (SnRK2s) and the ABA-responsive element (ABRE) binding factors AREB/ABFs (Thalmann *et al.*, 2016). This mechanism appears to be conserved amongst eudicots, as ABREs are present in the promoter of *BAM1* orthologs genes (Thalmann *et al.*, 2016).

Despite its importance, ABA is not likely to be the only regulatory factor in the reprogramming of carbohydrate metabolism under stress. For example, Dehydration Responsive Element Binding protein 1A and 2A (DREB1A and DREB2A) also can induce the transcription of many starch-degrading genes (Maruyama *et al.*, 2009). Further experimentation is required to disentangle the regulatory network controlling the transcriptional changes of carbohydrate-related genes under abiotic stress.

It should be noted that it may not just be the gene expression pattern, but also the characteristics of the encoded enzymes that may distinguish them from each other. BAM1 and BAM3, two closely related glucan hydrolytic enzymes, have different biochemical properties. BAM3 is more active at low temperature and slightly acidic pH values, whereas BAM1 prefers higher temperature and more alkaline pH (Monroe *et al.*, 2014). As the temperature is higher during the day and the pH in the stroma increases with the concomitant activation of photosynthesis, BAM1 and BAM3 may have undergone differential molecular evolution to fulfill their respective functions. Furthermore, many enzymes involved in starch metabolism are target of post-translational modifications, such as disulphide-bridge formation, phosphorylation or complex formation (reviewed by Kötting *et al.*, 2010). Of interest here is that both BAM1 and AMY3 are reversibly inactivated by disulphide-bridge formation (Sparla *et al.*, 2006; Seung *et al.*, 2013). However, the significance of this post-translational modification in plant stress tolerance is unknown.

VI. Conclusions and outlook

Starch metabolism is emerging as a key determinant of plant fitness under adverse conditions. The importance of starch during abiotic stress is well-documented in several plant species, but the overall available knowledge is still fragmentary, hampering our full understanding. To pick just one example, it is not obvious why in some cases starch content increased in response to high salinity. Is it a species-specific feature or simply a result of the experimental setup? A more consistent view of the changes in starch metabolism during abiotic stress will require experiments conducted under very similar conditions to enable direct comparisons between species and different kind of stress (Box 2).

Studies in Arabidopsis have been instrumental in revealing some of the underlying mechanisms and enzymes of the stress-induced starch degradation. However, there are likely to be more starch-degrading enzymes involved in this process than those identified so

Box 2 What is stress?

There is no commonly accepted definition of stress and consequently also no universal protocol for inducing stress. Generally, drought stress is induced using three major methods: by withholding watering; by reducing watering to a certain percentage of control; or by lowering soil water content or water potential to a certain value. Osmotic stress can be induced using three different osmolytes – mannitol, sorbitol and polyethylene glycol (PEG) – with concentrations ranging from 25 to 500 mM. For stress salt experiments, the concentration of NaCl can also vary from 25 to 600 mM. For cold stress induction, temperatures are generally lowered to 2–6°C, whereas heat stress is imposed by increasing temperatures to 25–40°C. The duration of stress also greatly varies from a few hours to several months. This enormous variation in the approaches used to induce the stress likely accounts for some of the differences seen between different studies. For future experiments, it is important to define homogenous treatments for stress induction in order to obtain more consistent results, allowing meaningful comparisons between different species or stresses.

far. For example, transcriptomics studies have reported stress-induced upregulation of genes coding for debranching enzymes, but their function in plant stress responses has not been investigated. It will be important to test the corresponding Arabidopsis mutants for their tolerance to abiotic stress. Furthermore, future research should be directed towards the understanding of how post-translation modifications of starch-degrading enzymes affect stress-induced starch degradation.

Lastly, with the knowledge gained from Arabidopsis, it will be possible to move forward with research and initiate improvements in plants of economic importance. Given the critical function of starch in plant stress responses, starch-degrading enzymes represent ideal targets for breeding stress-tolerant crops.

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6 – Composition and evolution of β-amylases in land plants

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Manuscript in preparation

Synopsis: As we had shown in the first chapter BAM1 is essential for stress-induced starch degradation in Arabidopsis. Here we show that BAM1 is conserved among all seed plants, and could serve a similar function in these plants. Interestingly, our analysis also revealed clear differences between species with regards to other BAM isoforms. On one hand we identified a novel isoform that was lost in all Brassicaceae (including Arabidopsis). On the other hand we found that BAM4 – an essential protein for nocturnal starch degradation in Arabidopsis – is absent in monocots, lamiids and legumes. Taken together our analysis provides a comprehensive overview of BAMs and their evolution in seed plants.

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Composition and evolution of beta-amylases in land plants

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Abstract

β-Amylases are key enzymes of plastidial starch turnover and have been extensively studied in the model plant *Arabidopsis thaliana*. However, little is known about their function in other species. To determine the level of conservation amongst different species we analysed the β-amylases of 115 land plants. Despite overall high conservation, our analysis revealed clear differences between Arabidopsis and many crop plants. Notably, a novel subgroup of β-amylases which is absent in Arabidopsis was found in virtually all angiosperms and gymnosperms, except the pinaceae family. Conversely, we found that BAM4 – an essential protein in Arabidopsis – is not found in many important crops species, including all cereals. Moreover, duplications of β-amylases were observed in many species or even whole families, indicating sub- and neofunctionalisation of those genes. Our work highlights the need to confirm results gained in model plants in other species, but also the possibility to do so using the wealth of genomic sequences available.

Introduction

β-Amylases (EC 3.2.1.2) are hydrolytic enzymes cleaving α-1,4 glucosidic bonds of polyglucan chains at the non-reducing end releasing maltose. They are found in all three domains of life. Amongst eukaryotes, they are absent in fungi and animals (Ophistokonta) but present in most other clades including plants (Archaeplastida). Previous research has shown that plant β-amylases (BAM) are derived from the eukaryotic host, not the cyanobacterial endosymbiont which gave rise to the plastid (Deschamps et al., 2008). Interestingly, the BAM family in land plants contains not only active enzymes, but also several catalytically inactive paralogs(Fulton et al., 2008), including two transcription factors (Reinhold et al., 2011). Further analysis of the β-amylases from land plants indicated that they could be subdivided in four subfamilies (Fulton et al., 2008). However, due to the sparse taxon sampling used in these analyses, the exact pattern of the expansion of the BAM family is still unclear.

Since the early age of molecular evolutionary genetics (Stephens, 1951; Ohno, 1970) gene duplication has been seen as an important source of variability available for evolutionary change. Indeed, the expansion of gene families due to the conservation of duplicated gene copies represents a fundamental factor in the generation of evolutionary novelties in many eukaryote lineages (Lynch et al., 2000). Whole genome duplication events (WGDs) have been identified as an important contributor to the expansion of gene families in many groups (Edger and Pires, 2009), including vertebrates (Panopoulou and Poustka, 2005; Marlétaz et al., 2015) but especially plants (De Bodt et al., 2005). Even if most of the duplicated gene copies generated by a WGD are lost (De Smet et al., 2013), the sub- or neo-functionalization of the gene copies leads to an increase in the gene number, driving the diversification of gene families (Panchy et al., 2016; Hammoudi et al., 2016).

To identify the pattern of duplication, gene loss and function of BAMs among land plants, we retrieved genes from over one hundred species using publically available data bases. To infer the function of newly identified genes we by compared them to the well-studied Arabidopsis and soy bean orthologs.

The crystal structure of soybean β-amylase revealed that the hydrolysis of the glucosidic bond is catalysed via a general acid-base catalysis mechanism by two glutamic acid residues (Mikami et al., 1994). In the soybean enzyme, Glu-186 acts as a general acid while Glu-380 acts as a general base (Kang et al., 2005; Mikami et al., 1994). The carboxyl group of Glu-186 is located on the hydrophilic surface of the glucose and protonates the glucosidic oxygen. Subsequently, the deprotonated Glu-186 is stabilized by Thr-342 located at the inner loop (Kang et al., 2005). The carboxyl group of Glu-380 lies on the hydrophobic face of the glucose residue at the subsite −1 and activates the attacking water molecule, which ultimately leads to the cleavage of the glycosidic bond. In addition to those three regions directly involved in the catalytic reactions a fourth region – the flexible loop – corresponding to amino acids 96-103 of the soybean enzyme, is essential for binding of the glucan chain and enzymatic activity. The reducing glucose of the released maltose is in the β-form, explaining the name β-amylase.

While the crystal structure was solved in soybean, most studies investigating the function of β-amylases *in vivo* were conducted in the model plant *Arabidopsis thaliana*. The genome contains nine genes encoding β-amylase-like proteins (Table 1). At least four β-amylases (BAM1 to BAM4) are targeted to the chloroplast (Fulton et al., 2008); two more (BAM7 and BAM8) are nuclear proteins (Reinhold et al.,

2011) while BAM5 is a cytosolic protein and mainly localized to sieve elements in the phloem (Wang, 1995). To this date there has been no detailed investigation of AtBAM6 and AtBAM9 and their physiological function.

Gene	AGI code	Amino acids	Active	Demonstrated localisation	References
BAM1	At3g23920	575	Yes	Chloroplast	(Kaplan and Guy, 2005; Valerio et al., 2011; Horrer et al., 2016; Thalmann et al., 2016)
BAM2	At4g00490	553	low	Chloroplast	(Fulton et al., 2008)
BAM3	At4g17090	548	Yes	Chloroplast	(Fulton et al., 2008; Kaplan and Guy, 2005)
BAM4	At5g55700	531	No	Chloroplast	(Fulton et al., 2008)
BAM5	At4g15210	498	Yes	Cytosol	(Laby et al., 2001; Wang, 1995)
BAM6	At2g32290	577			
BAM7	At2g45880	691	No	Nucleus	(Reinhold et al., 2011; Soyk et al., 2014)
BAM8	At5g45300	689	No	Nucleus	(Reinhold et al., 2011; Soyk et al., 2014)
BAM9	At5g18670	536			

Table 1: AGI gene codes and known information for the Arabidopsis β-amylase gene family.

Several β-amylases are key enzymes of plastidial starch degradation. This is illustrated by the starch excess phenotype of Arabidopsis plants lacking chloroplastic β-amylase isoforms (Fulton et al., 2008; Kaplan and Guy, 2005) as well as by the rapid accumulation of the their product maltose during the night when starch is degraded (Fulton et al., 2008; Niittylä et al., 2004). Of the four β-amylases known to localise to the chloroplast BAM1 and BAM3 are both active enzymes and recombinant proteins have high specific activities on glucan substrates *in vitro* (Fulton et al., 2008). Conversely, BAM2 has a very low specific activity while BAM4 appears to be non-catalytic due to amino acid substitutions within its active site, including one of the two catalytic glutamate residues (Fulton et al., 2008).

Mutants of *BAM3* have a mild starch excess phenotype whereas mutants of *BAM1* show no obvious alteration in leaf starch metabolism compared to wild-type plants (Fulton et al., 2008). Additionally, BAM3 has been implicated in cold-stress induced starch degradation (Kaplan and Guy, 2005). On the other hand AtBAM1 has been found to be involved in starch degradation in guard cells during stomatal opening (Horrer et al., 2016), resistance to osmotic stress (Valerio et al., 2011; Thalmann et al., 2016; Monroe et al., 2014; Zanella et al., 2016) and heat stress (Kaplan and Guy,

2004). Despite these specialisation there is partial overlap in the functions of BAM1 and BAM3, as the *bam1bam3* double mutant has a stronger starch excess phenotype than the *bam3* single mutant (Fulton et al., 2008). Thus, it appears that AtBAM3 is the major isoform during night-time starch degradation, but AtBAM1 can also contribute to this process.

Although the BAM4 protein has no detectable β-amylase activity, the *bam4* mutant in Arabidopsis does have a *starch excess* phenotype. It is unclear how a non-catalytic β-amylase-like protein could influence starch breakdown. It has been speculated that AtBAM4 could act as a chloroplastic regulator, potentially responding to the concentration of maltose, and thereby fine-tuning the rate of starch degradation (Fulton et al., 2008) or as a scaffold protein facilitating the binding of other degrading enzymes to starch (Li et al., 2009). However, direct evidence for either function is lacking.

To date no role for BAM2 is known. Although it is an active enzyme, no change in phenotype could be observed when BAM2 was missing either alone or in combination with other β-amylases; and it has been speculated that BAM2 is the result of a duplication of BAM7 followed by a partial gene deletion (Fulton et al., 2008).

Not all β-amylases are involved in starch metabolism: it was shown that two of them (BAM7 and BAM8) are localised to the nucleus and possess an additional Brassinazole resistant 1 (BZR1)-type DNA binding domain (Reinhold et al., 2011). These proteins act as transcriptional regulators controlling shoot growth and development by interacting with brassinosteroid signalling, but have no direct influence on starch degradation. It was suggested that the β-amylase-like domain could act as a metabolite sensing domain rather than catalysing the hydrolysis of glucans like true β-amylases (Reinhold et al., 2011). Further evidence for this model is provided by Soyk et al. (2014): it was shown that eradicating the residual enzymatic activity by the substitution of Glu-429 of AtBAM8 in Arabidopsis (corresponding to Glu-180 in soybean) led to no change in the transcription factor activity. In contrast, the amino acid substitution of Glu-623 (Glu-380 in soybean) which prevents substrate binding caused a drastic reduction of the transcriptional activator function of AtBAM8.

The cytosolic AtBAM5 appears not to be involved in starch breakdown either, as the corresponding *bam5* mutants have normal starch levels (Laby et al., 2001).

In contrast to the detailed analysis performed in Arabidopsis little is known about the physiological role of β-amylases in most other plants, including most crop species. However, the existing data indicates that they play an important role in plastidial starch turnover in rice (Hirano et al., 2016) and potato (Scheidig et al., 2002). As starch degradation is an important

The aim of this work was to use the wealth of genomic sequences available to determine to what extent the β-amylases found in *Arabidopsis thaliana* are conserved in other plant species focusing on land plants (embryophytes). Furthermore, we investigated to what extent duplications and neofunctionalisations contributed to the emergence of novel BAM isoforms during the evolution. To address these questions we used both genomic and transcript sequences to analyse the composition, evolution and properties of the β-amylase family of land plants. We identified eight distinct β-amylase clades and characterized the origin and loss of each protein throughout the evolution of different lineages of seed plants.

Methods

Genomic sequences and resources

Protein sequences of β-amylase were retrieved from genomes available in the current NCBI Entrez Genome Project, Phytozome, and the websites of authors. Additionally, β-amylase sequences from gymnosperms, ferns, basal embryophytes and charophytes were retrieved from the transcriptomes available in the OneKP database (See supplementary table S1).

Multiple sequence alignment and phylogenetic analysis

Alignments of protein sequences were performed using ClustalW (Thompson et al., 1994) and Mafft (Katoh and Toh, 2008). Phylogenetic analysis was performed in MEGA ver 6 (Tamura et al., 2013). A Neighbor Joining starting tree was built built using a JTT model, and optimized by a Maximum Likelihood using Subtree Pruning-Regrafting as search method. Branch support was calculated on 500 bootstrap replicates.

Prediction of subcellular localisation

Transit peptides were predicted using ChloroP1.1(Emanuelsson et al., 1999), nuclear localisation signals were predicted using NLStramadus (Nguyen Ba et al., 2009). As the transit peptide is always at the very N-Terminus of a protein and the nuclear localisation signal in Arabidopsis BAM7 and BAM8 is likewise found in the N-terminal

part of the protein (Reinhold et al., 2011), only protein sequences covering the N-terminus were included into this analysis.

Results

Eight distinct BAM clades were already present in the ancestor of the flowering plants

Previous phylogenetic analysis has identified four distinct subfamilies of β-amylases (Fulton et al., 2008). Our more exhaustive analysis allows us to subdivide these subfamilies into eight distinct clades, which are conserved in virtually all angiosperms (Fig 1). Clade I contains AtBAM1 and its orthologs. Clade II is composed of a previously unidentified β-amylase isoform without an ortholog in Arabidopsis. Clade III consists out of AtBAM3 and its orthologs. The branch separating these three clades from the rest of the BAMs receives strong bootstrap support (86). Clades IV and V contain the orthologs of AtBAM4 and AtBAM9, respectively. The grouping of these two orthologs in a clade does not receive strong bootstrap support in our analysis (52, Fig 1). Clade VI contains both AtBAM5 and AtBAM6 as well as their orthologs. Clade VII contains AtBAM2 and AtBAM7 together with their orthologs, while clade VIII contains AtBAM8 and its orthologs. The monophyly of Clade VII and VIII is strongly supported by our analysis (boostrap 80 and 98 respectively, Fig 1).

BAM sequences representing each of the eight clades were found in genomes of most sequenced angiosperms indicating that the eight β-amylase clades were present in the ancestral genome of flowering plants and subsequently maintained in most of the extant representatives of the angiosperms. Clade VII and VIII appear to be angiosperm-specific as no sequences belong to either clade were found in other seed plants. Indeed, grouping sequences from gymnosperms with clade VIII receives very low bootstrap support, and most sequences from gymnosperms, lycophytes and mosses subtend the split of clade VII and VIII suggesting that these two clades originated after the separation of the angiosperms from the rest of the seed plants.

The different clades emerged during the evolution of land plants but are absent in green algae

Orthologs of the remaining six clades as well as at least one copy of the BZR-BAMs were found in genomes and transcriptomes of most analysed gymnosperms. Conversely, the moss *Physcomitrella patens* and the lycophyte *Selaginella moellendorfii* contain only an ancestral BZR-BAM as well as an isoform subtending the split of Clades I-III (Fig 2). However, orthologs of clade V and VI were found in the transcriptome of other lycophytes and also in liverworts, while orthologs of clade IV were identified in the transcriptome of hornworts and liverworts and ferns (Fig 2).

In contrast orthologs of clades I-III can only be found in seed plants, while sequences from basal land plants attach to the branch subtending the split between these three clades. Taken together, our results place the emergence of clade IV, clade V, and the ancestral BZR-BAM before the radiation of land plants, while clade I, II and III developed only later but before the emergence of seed plants.

Green algae closely related to land plants (charophytes) contain unique algal BAMs which share little similarity with land plants. However, one isoform is sister to a clade comprising BAM1, BAM3 and BAM10, indicating that these three forms probably derived from a single protein ancestor before the origin of land plants. Likewise, another charophyte BAM is related to clade VI.

β-amylases in chlorophytes are only distantly related to embryophyte BAMs and form several distinct clades.

Activity but not localisation is conserved in each clade
Comparison of residues required for catalytic activity amongst different orthologs showed that they are highly conserved among orthologs of AtBAM1 and AtBAM3 which are known to be active (Fig 4), suggesting that all orthologs are also active enzymes. Conversely, catalytic residues were much less conserved in isoforms whose Arabidopsis orthologs were shown to be inactive such as BAM4, BAM7 and BAM8. In particular the inner loop, the catalytic residue Glu-380 and its surroundings were poorly conserved in all three clades. However, the second catalytic residue (Glu-186) was conserved even in inactive proteins indicating that it is required not just for catalytic activity but also has other functions. Interestingly, while the flexible loop was heavily mutated in BAM4 orthologs, it is still largely conserved in BAM7 and BAM8. In all cases our results are in line with the demonstrated activity of the respective Arabidopsis orthologs.

We also analysed catalytic residues of BAM6 and BAM9 which have not been investigated *in vitro* to date. Our analysis revealed that all residues important for catalysis are conserved in BAM6 which thus appears to be active, while BAM9 contains numerous mutations and deletions in key residues, likely leading to an inactive protein (Fig 4).

In silico predictions indicate that most isoforms of a clade I, IV, and VIII localise to the same compartment as their Arabidopsis orthologs (Table 2, Supplementary table 2). Clade VII contains two Arabidopsis orthologs, BAM2 and BAM7. While BAM2 has been shown to be a plastidial protein (Fulton et al., 2008), BAM7 is a nuclear protein (Reinhold et al., 2011). Most orthologs are predicted to localise to the same

compartment, i.e. BAM2 orthologs to the plastid and BAM7 orthologs to the nucleus, showing that the localisation can change within a clade. Most members of Clade VI are like AtBAM5 predicted to be cytosolic proteins, but orthologs of AtBAM6, which form a Brassicacea-specific subclade, are predicted to be plastidial proteins.

No clear prediction of localisation could be obtained for clade III and V. While AtBAM3 is a plastidial protein (Fulton et al., 2008), only 55% of the other Clade III proteins are predicted to share this localisation. Predictions of the localisation of clade V are likewise inconclusive, 45% of the sequences are predicted to contain a transit peptide, while the remainder doesn't. It is unclear whether this uncertainty is due to limitations of the bioinformatical tools or reflects a genuine change in subcellular localisation of different orthologs.

Clade	Arabidopsis orthologs	Predicted localisation
I	BAM1	Plastidial (68/92)
II	-	Plastidial (40/40)
III	BAM3	Plastidial (45/82)
IV	BAM4	Plastidial (22/30)
V	BAM9	Cytosolic (44/77)
VI	BAM5	Cytosolic (63/77)
	BAM6	Plastidial (7/11)
VII	BAM2	Plastidial (35/39)
	BAM7	Nuclear (42/49)
VIII	BAM8	Nuclear (49/49)

Table2: Predicted localisation of proteins from different clades.

An additional BAM isoform was identified

Our analysis revealed a novel clade of β-amylases (clade II), containing isoforms which we named BAM10. BAM10 is not found in Brassicales (including the model plant *Arabidopsis thaliana*) but orthologs are present in most other Angiosperms. Amongst gymnosperms it is notably absent in Pinaceae, but sequences of BAM10 were retrieved from the transcriptomes of members of the Cupressophyta (*sensu* Cantino et al., 2007). Partial BAM10 sequences were also identified in *Ginkgo biloba*, *Welwitschia mirabilis*, as well as in cycads, indicating that BAM10 emerged before the radiation of seed plants (Fig 3A). Analysis of the expression profile of the tomato orthologs indicates that it is expressed in most tissues (Fig 3B). All BAM10 orthologs are predicted to localise to the plastid (Table 2).

Alignment of BAM10 sequences with active (BAM1 and BAM3) and inactive (BAM4) β-amylases shows that BAM10 carries numerous mutations which would likely result in an inactive protein (Fig 4). In particular, the flexible loop region of β-amylases, which is known to be crucial for the formation of the substrate tunnel and binding of glucan (Mikami et al., 1994), is conserved in BAM1 and BAM3 orthologs as

GGNVGD but contains numerous mutations in BAM10 genes. Likewise, the inner loop is poorly conserved, with Thr-342 substituted with serine in many genes. Thr-342 normally interacts with the catalytic Glu-186 and the glucan substrate, and its substitution to serine results in a 360-fold reduction of k_{cat} in GmBAM1 (Kang et al., 2005). Furthermore, while the catalytic residue Glu-380 is conserved in a majority of BAM10 genes, the surrounding amino acids are poorly conserved. In all these regions important for catalysis, BAM10 more resembles the inactive BAM4 than the active BAM1 and BAM3. Thus, despite the overall similarity of BAM10 to BAM1 and BAM3, it appears to be a catalytically inactive protein.

All analysed BAM10 orthologs are predicted to be plastidial proteins.

BAM4 is absent in many species

Despite being inactive, BAM4 is play an important regulatory role in leaf starch degradation in Arabidopsis. Mutants lacking BAM4 have a starch excess phenotype (Fulton et al., 2008). Our analysis revealed that orthologs of AtBAM4 are not found in any monocotyledon species, and are likewise absent in Fabaceae and Lamiids (Fig 5). These groups include many important crop plants including all major sources of starch except cassava (*Manihot esculenta*). In addition to these three large families, BAM4 is also not found in Salicaceae, *Citrus* and *Eucalyptus*, indicating that it might have been lost many times in the evolutionary history of the angiosperms. Given that AtBAM4 is essential for Arabidopsis leaf starch breakdown, it is surprising that BAM4 is so poorly conserved in other plants. This suggests that there may be a higher degree of diversity in the mechanism of starch degradation between leaves of different species than previously thought.

BZR-BAMs likely formed in early land plants through the fusion of a β-amylase with a novel BZR/BES-like protein

AtBAM7 and AtBAM8 are unique amongst Arabidopsis BAMs as they contain in addition to the β-amylase domain a second domain resembling the BZR1/BES1-type transcription factors, and are thus named BZR-BAMs. We tried to elucidate the origin of this second domain by searching for BRZ-like proteins in land plants. We identified a class of BES1/BZR1-type transcription factors in bryophytes which is most similar to the BZR-domain of BAM7 and BAM8. The novel genes fall in a separate clade from the remaining BES1/BZR1-type transcription factors and are absent in higher plants (Fig 6).

Thus, it seems that members of the Clades VII and VIII have acquired their additional (BZR) domain from the BZR1-type transcription factor present in the basal land

plants, which had already diverged from the remaining BES1/BZR1-type transcription factors before the fusion event.

In some members of Clade VII the BZR domain is absent, e.g. in AtBAM2. As those genes are nested within BZR containing orthologs, they represent the outcome of a secondary loss of the BZR domain. The different BAM2-like (i.e. BZR-less) genes do not form a separate clade. Indeed, the BAM2-like protein of grasses are more related to the BAM7 of grasses than the BAM2-like genes of dicots. Therefore, it appears that BAM2-like genes represent a polyphyletic assemblage of proteins independently generated by subfunctionalization of BAM7 orthologs by loss of the BZR-like domain.

Duplications of β-amylases occurred frequently in the evolution of land plants
Our findings indicate that the emergence of new β-amylase isoforms through gene duplications has been a common occurrence in the evolution of angiosperms. Previous work suggests that BAM2 and BAM7, as well as BAM5 and BAM6, are paralogs (Fulton et al., 2008). Our analysis confirms and extends this observation. BAM6 appears to have originated after a recent duplication specific for the Brassicales as it is not found outside of this family. Interestingly, while BAM5 is known to be a cytosolic protein (Laby et al., 2001) and most genes in clade VI likewise lack a transit peptide, most BAM6 orthologs are predicted to be localised in the chloroplast (Table 2). As the BAM6 orthologs are nested within Clade VI, this suggests that the transit peptide of AtBAM6 evolved after its duplication. BAM2-like proteins of Brassicales form a well-supported clade which is derived from a duplication of BAM7 followed by a deletion of the BZR-domain. Other Rosid species also contain BAM2-like proteins but they do not cluster with Brassicales BAM2s. As mentioned above, other BAM2-like proteins also formed independently in monocotyledonous species.

In addition to the duplications giving rise to AtBAM2 and AtBAM6, numerous other duplications of β-amylase genes were identified. The genomes of grasses encode two paralogs of BAM1, BAM5, and BAM9. Interestingly enough, one of the two paralogs in Clade VI is –like the Brassicales BAM6 – predicted to localise to the chloroplast. As these duplications are clearly found in all Poaceae they most likely arose during the ancestral whole-genome duplication proposed by Salse et al., 2008. Likewise, the duplication of BAM5 found in legumes appears to be the result of a paleopolyploidy of the ancestral legumes (Shoemaker et al., 2006).

Aside from these duplications conserved in whole families, duplications present in only certain species were identified. Some are linked to recent polyploidization

events: the hexaploid *Camelina sativa* (Kagale et al., 2014) contains three copies of most β-amylases, while the amphidiploid *Brassica napus* (Rana et al., 2004) contains two.

Discussion

In the present study, we examined β-amylase homologs in 115 land plant species, including 66 sequenced genomes. The number and diversity of organisms examined in this study should be considered sufficient to identify the main patterns of β-amylase evolution in land plants. Although ongoing plant genome projects will certainly uncover additional species- or family-specific deletions and duplications, the general features is likely to not change.

Across all spermatophytes we identified 7 clades of β-amylases, one of which underwent a duplication in the ancestor of angiosperms giving rise to Clades VII and VIII. The sequenced genomes of *P.patens* and *S.moellendorfii* contain only genes encoding for the ancestral BZR-BAM and the progenitor of Clade I-III, but interestingly orthologs of other clades are found in the transcriptome of other basal land plants. This indicates that at least some additional clades were present in the ancestor of all land plants. Their absence in *P.patens* and *S.moellendorfii* is likely the result of either annotation problems or species-specific deletions. On the other hand, green algae lack clear orthologs of most identified clades identified in seed plants. Taken together our results show the divergence of β-amylases clearly precedes the emergence of seed plants but occurred after the colonisation of the colonisation of terrestrial habitats (Fig 7).

BZR-BAMs are present in basal land plants. Interestingly, the full brassinosteroid signalling pathway is thought to have been acquired only in higher plants, and the functional brassinosteroid receptor BRI1 is only found in flowering plants (Depuydt and Hardtke, 2011). However, BRI1-like genes and genes involved in brassinosteroid signalling are found in vascular plants (Vriet et al., 2015). The early emergence of BZR-BAMs indicates that the brassinosteroid signalling pathway was already partially functional in early land plants. Alternatively, the BZR-BAMs might have originated independently of the brassinosteroids and were later recruited to the pathway. Further work will be required determine the function of BZR-BAMs in bryophytes and their relation to brassinosteroids. Nonetheless, it is interesting that the duplication of the BZR-BAMs in flowering plants coincides with the emergence of functional BRI1-receptors.

Of all clades, clade IV is the least conserved clade being absent in over half of the analysed species. In *Arabidopsis* AtBAM4 is essential for night-time starch degradation as demonstrated by the starch-excess phenotype of the corresponding mutant (Fulton et al., 2008). However, our analysis shows that BAM4 is absent in many species, including cereals and potatoes, which are the major source of starch both for industrial application and nutrition. Furthermore, several other important crops like soybean, coffee and tobacco also lack BAM4. The absence of an essential protein in many different species indicates the presence of alternative pathways among flowering plants and that insights gained in Arabidopsis are not necessarily useful for biotechnological engineering in these species.

It is unclear why Clade IV appears dispensable in many species given its importance in *A.thaliana*. As AtBAM4 can efficiently bind starch, it is thought to allow more efficient binding of other starch degrading enzymes, although it is unknown how it participates in starch degradation (Li et al., 2009). Plants lacking BAM4 might rely on another β-amylase to mediate interactions between starch and degrading enzymes. A potential candidate is the newly discovered BAM10 as both enzymes are predicted to be inactive and localise to the plastid. Alternatively, the enzymes interacting with AtBAM4 might have adapted in these species to interact with starch directly, rendering BAM4 superfluous.

While BAM4 is absent in many species, we found a novel β-amylase which was lost in the Brassicales lineage. While BAM10 is most likely inactive it is nonetheless expressed in tomato and virtually all orthologs are predicted to localise to the plastid. Further work will be necessary to characterize the protein and its role in starch metabolism.

Clades I-III are only found in seed plants, however in genomes and transcriptomes all bryophytes, lycophytes and ferns at least one BAM gene was found that clustered with these clades (bootstrap support 66%). This indicates that the three clades only diverged after the radiation of vascular plants. In Arabidopsis BAM1 (Clade I) and BAM3 (Clade III) have clearly different functions. BAM1 degrades starch in guard cells and during osmotic stress, while BAM3 is responsible for night-time starch degradation in mesophyll cells. Interestingly, while stomata are present in basal land plants, unequivocal active control of stomatal movement is only found in seed plants, while stomata in ferns close more slowly if at all (Roelfsema and Hedrich, 2016). It is possible that the recruitment of a β-amylase for stomatal carbon metabolism imposed conflicting selection pressures on the ancestral BAM, which could be resolved by

duplication and subfunctionalisation. It would be interesting to investigate the function of the ancestral BAM with regard to mesophyll and guard cell starch metabolism in ferns.

In addition to the deletions we also identified numerous duplications. Indeed, over 60% of all analysed species had a duplication of at least one β-amylase gene. Several duplications are conserved across whole families, indicating sub- or neofunctionalisation. In some cases, the duplication involved a shift in the localisation of the proteins: both Brassicales and Poales carry two copies in Clade V, and in both families one isoform has become localised to the plastid while the other has remained cytosolic. The conservation of duplicated copies of many lineages of the BAM genes underlines the potential plasticity and flexibility of the starch degrading pathways, and opens the possibility of investigating the evolvability of this pathway at different evolutionary levels.

Concluding, in this work we carried out comprehensive analyses of β-amylases in land plants to better define their role. Our data provide comprehensive overview of composition and evolution of β-amylases in seed plants identifying 7 clades including one not described before. We further showed that while the divergence of the 7 clades is old, different clades have been lost repeatedly during the evolution of land plants, while duplications gave rise to new isoforms. Thus, our work highlights the potential of harnessing the publically available genome and transcriptomes to analyse the evolution of gene families and identify differences between species.

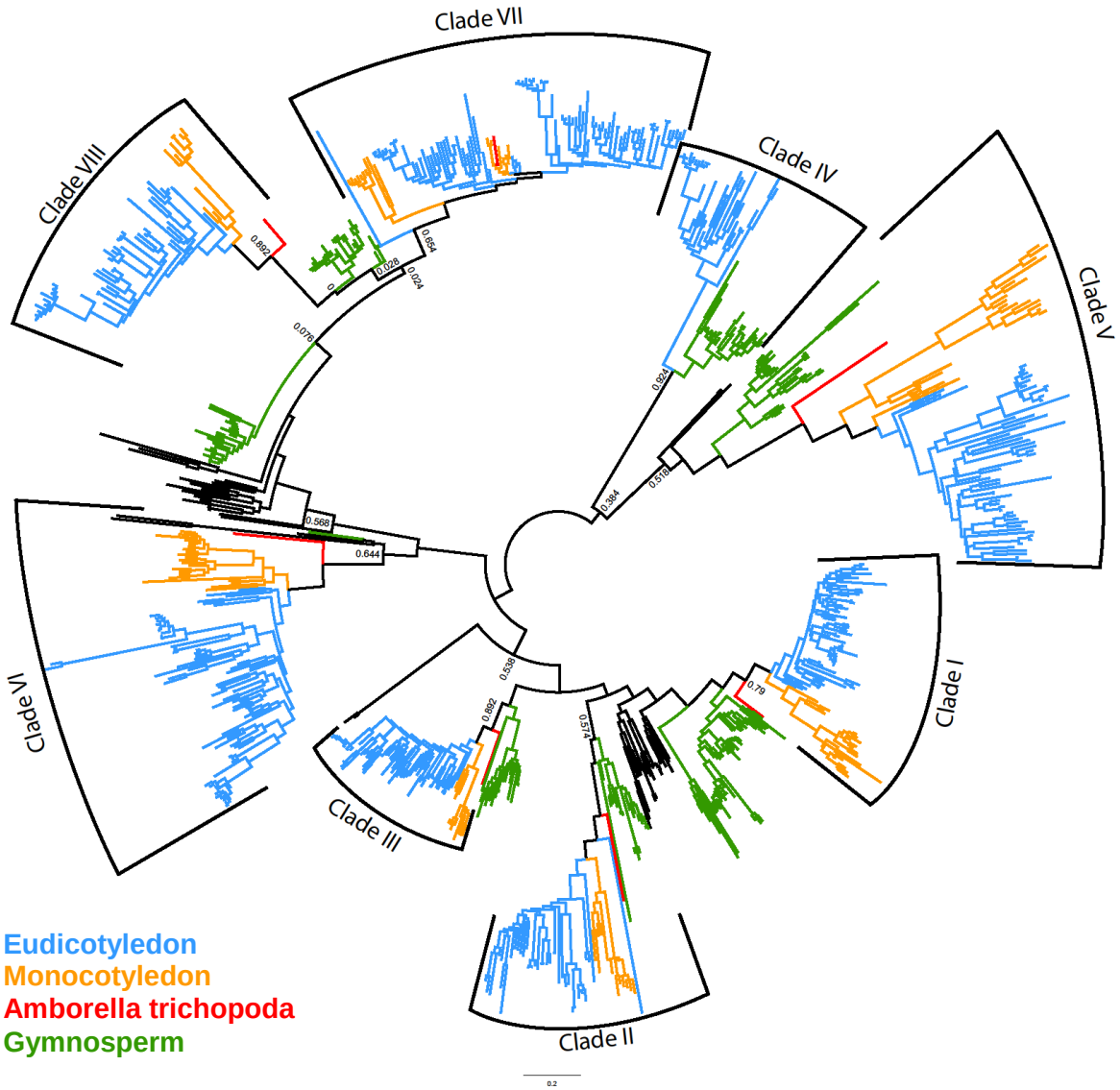


Figure 1: Evolution β-amylases in streptophytes. β-amylases from all spermatophytes fall into 8 clades. The divergence of Clade VII and VIII is only found in flowering plants, the orthologs of other land plants cluster to the base of both clade VII and VIII.

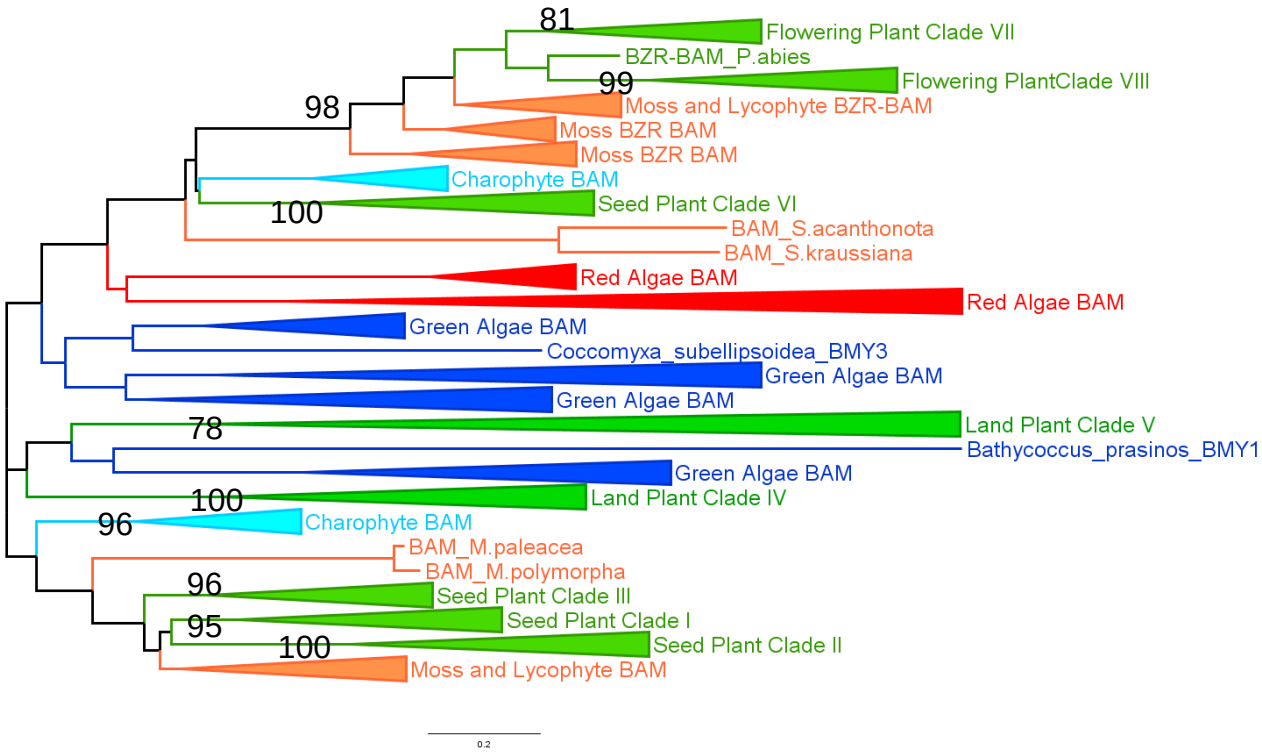


Figure 2: Analysis of β-amylases from basal embryophytes and selected spermatophytes (*A.thaliana, P.trichocarpa, S.lycopersicum, P.dactylifera, B.distachyon, A.trichopoda, P.abies*). Two large subfamilies were identified: Subfamily I containing Clade I, II and III as well as a clade specific to mosses and algae. Clade II and III are only found in spermatophytes, while Clade I is also present in lycophytes. Superfamily II contains Clade VII and VIII and the ancestral genes of mosses. It is only found in land plants. Clade IV, V and VI are not clearly placed and their origin cannot be determined. In addition to the 8 clades found in spermatophytes three clades specific to algae and mosses were found.

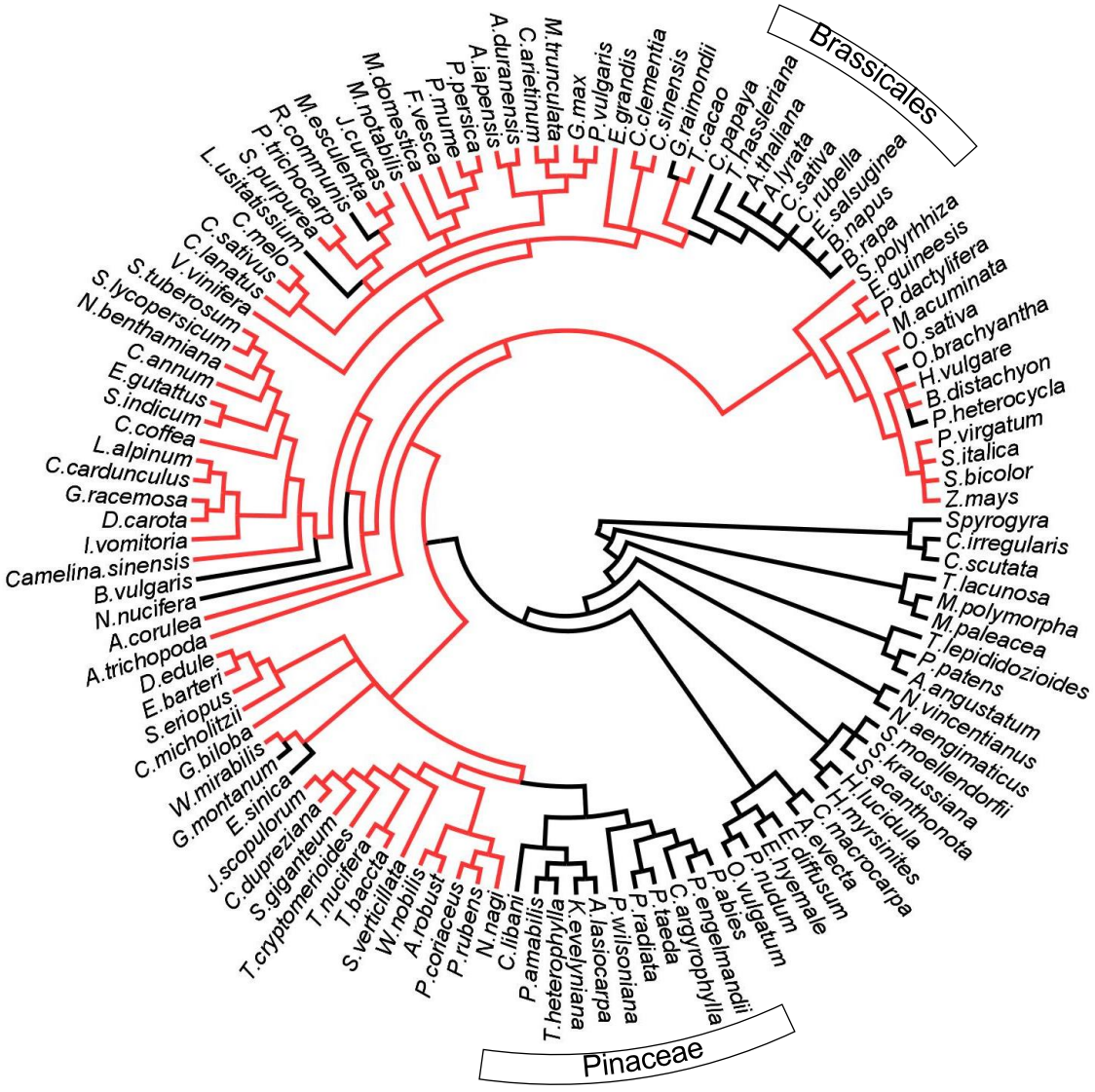
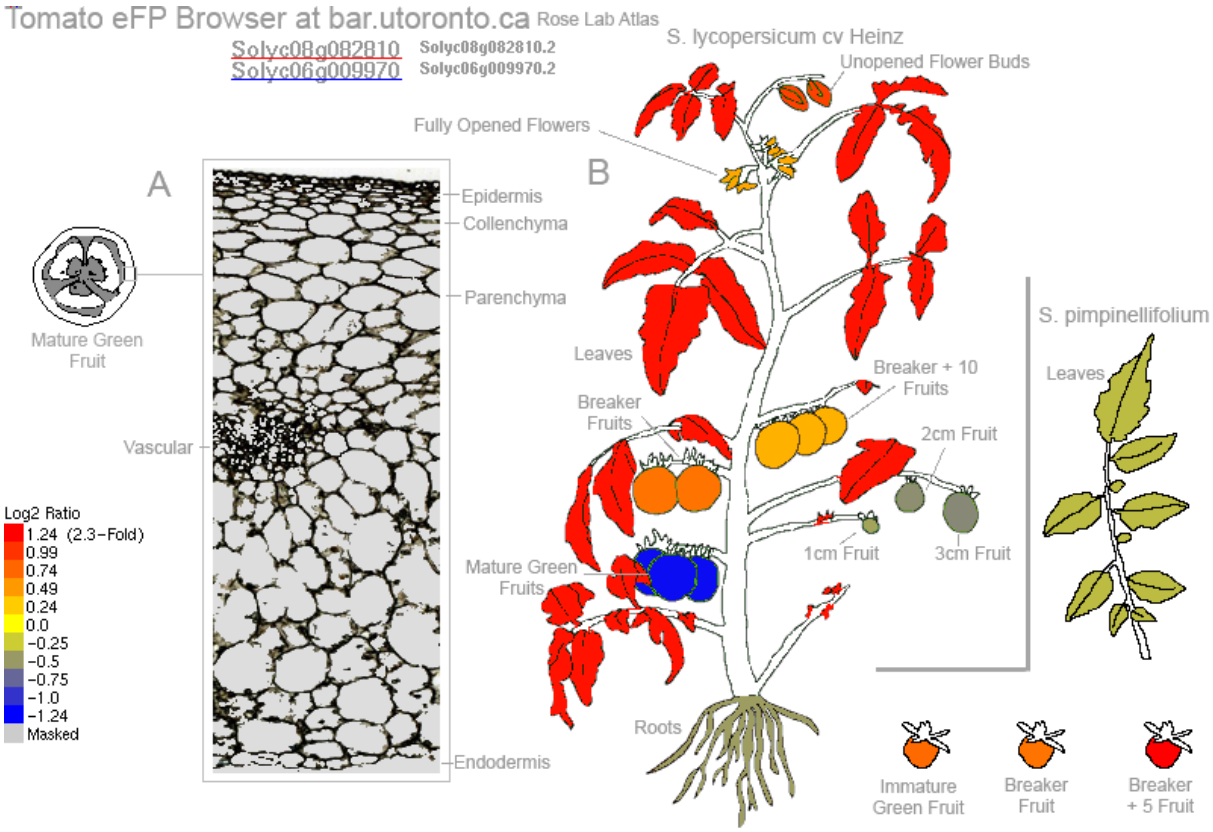


Figure 3: (A) A new clade of β-amylases was found in almost all spermatophytes, but is absent in conifers and brassicales.



(B) Expression data of BAM10 in tomato, expression is relative to Elf1α

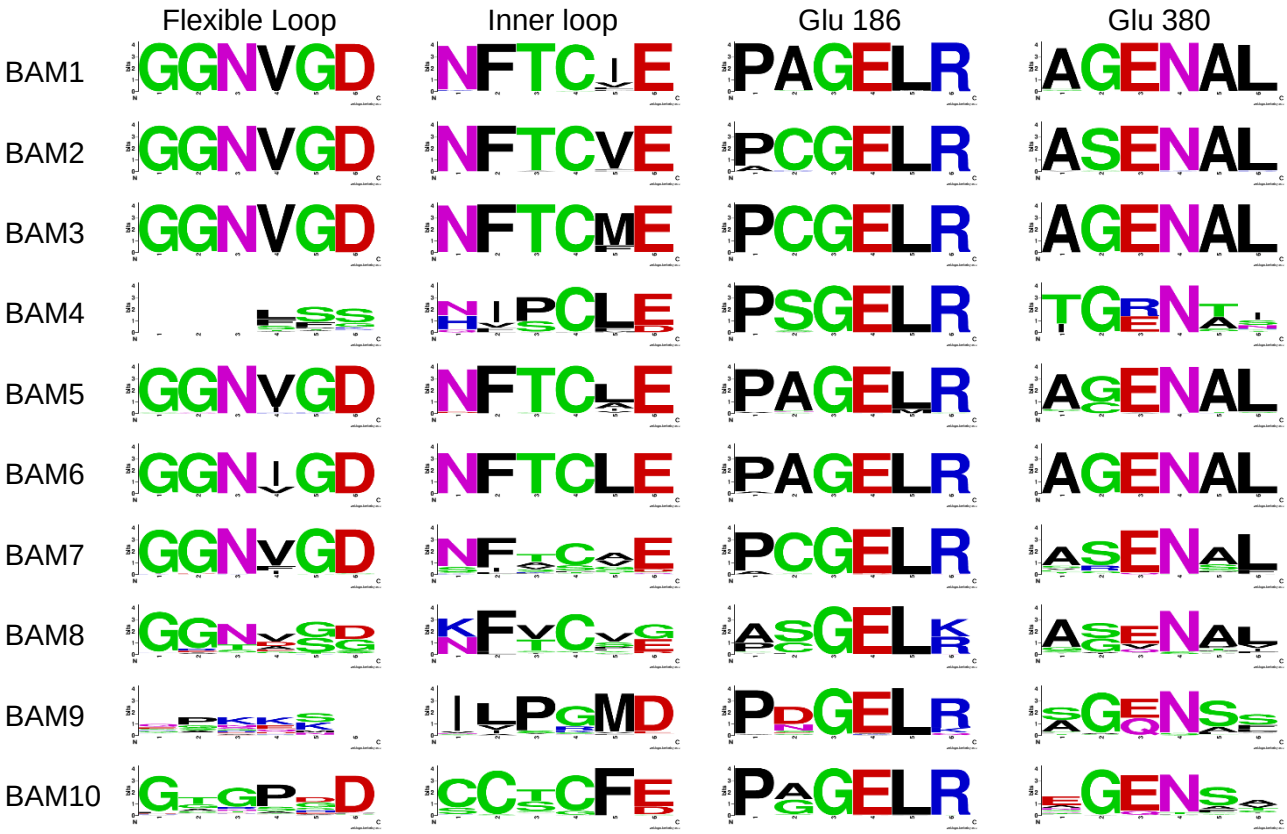


Figure 4: Comparison or residues important for catalytic activity. Note that while in the active BAM1 and BAM3 these residues are highly conserved, they are substituted in both BAM4 – which is known to be inactive – and BAM10. Flexible loop: amino acids 340-346, Inner loop: amino acids 96-103. Sequence Logos were made using WebLogo (http://weblogo.berkeley.edu/logo.cgi).

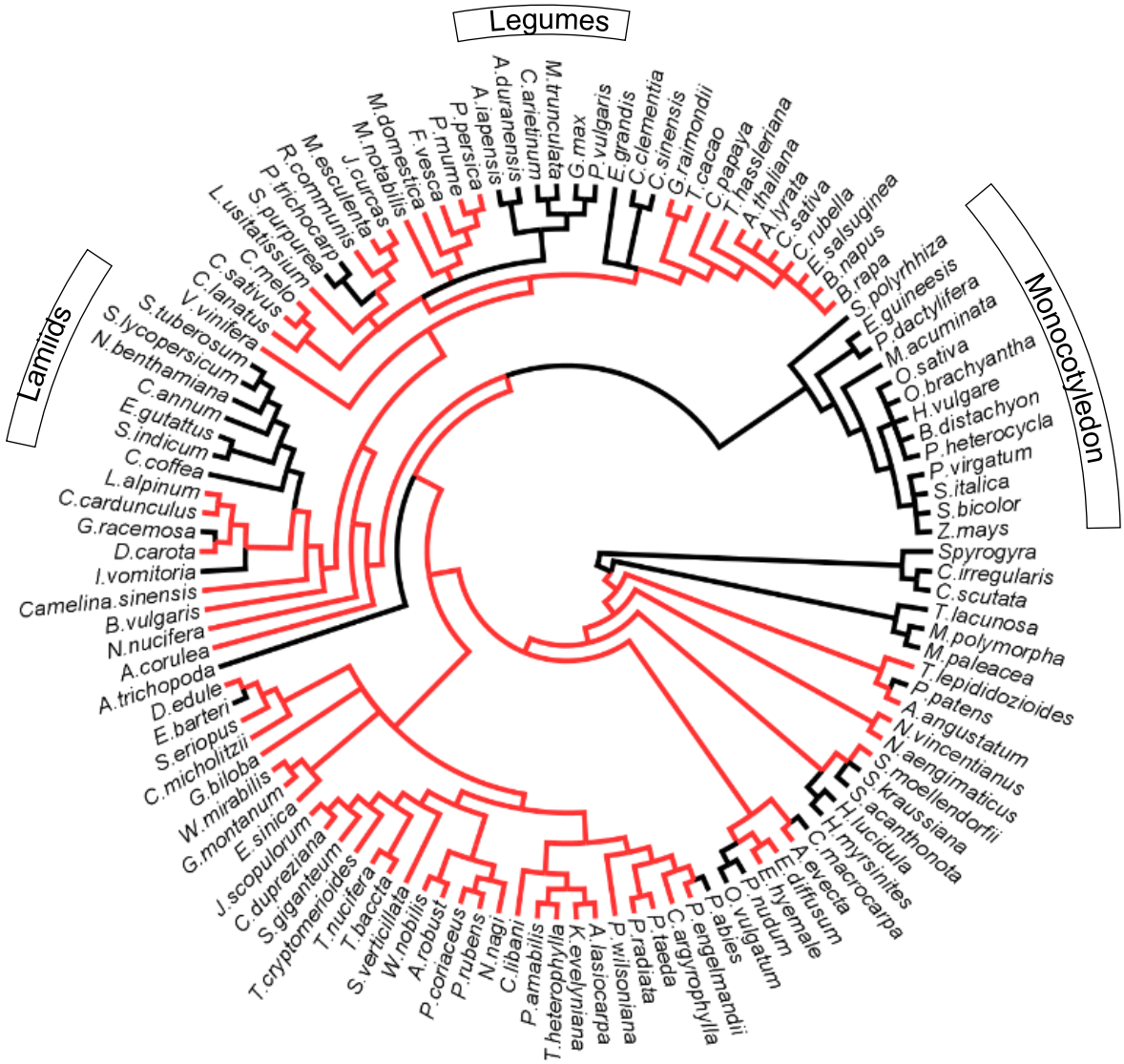


Figure 5: Orthologs of BAM4 are missing in many species, including all monocotyledon, fabaceae and lamiids. Nonetheless BAM4 was found in the transcriptome of hornworts and mosses indicating that it emerged early during the evolution of landplants

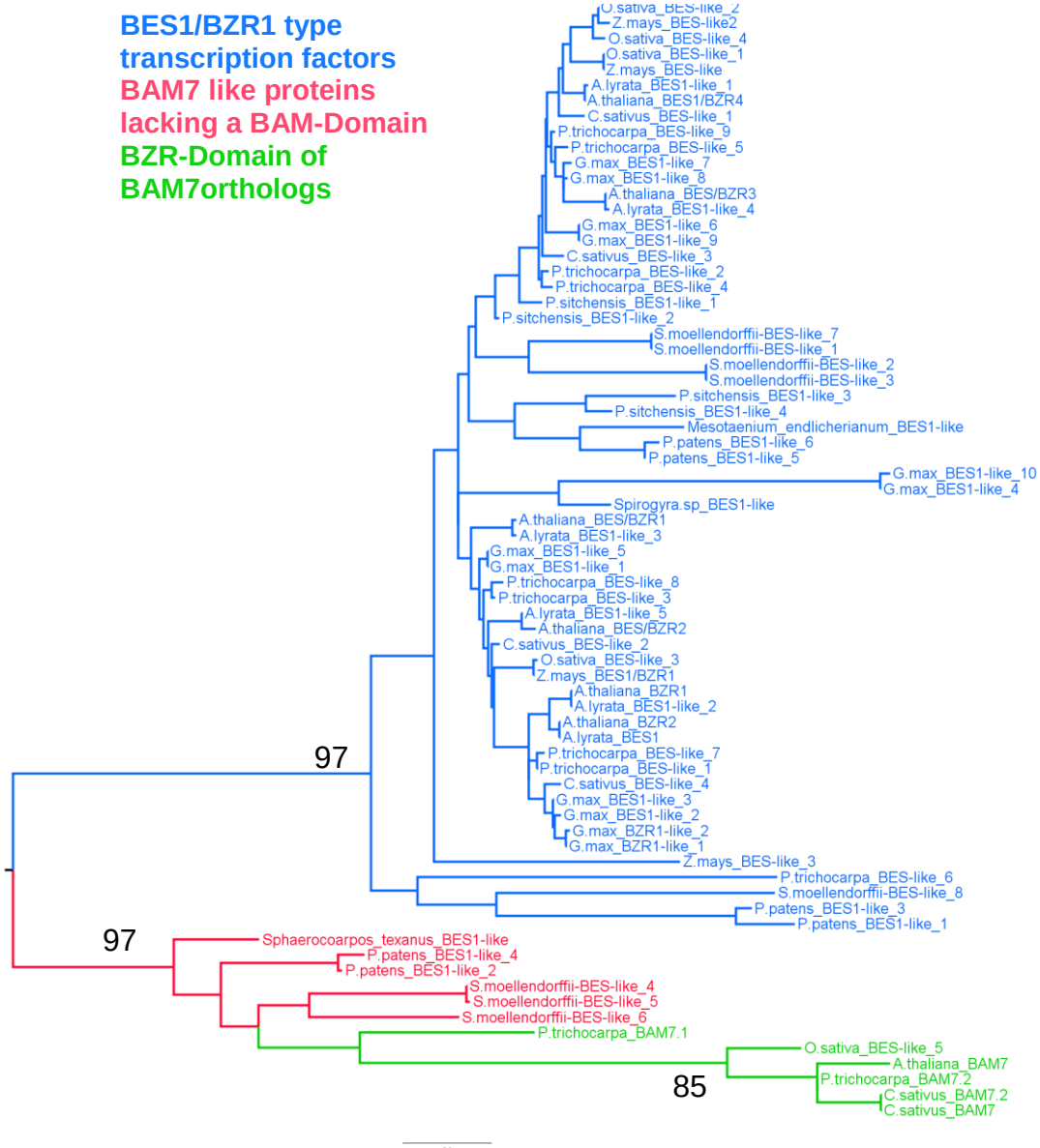


Figure 6: Phylogenetic relationship of BES1/BZR1-type transcription factors and the DNA-binding domain of BAM7. Basal plants contain an novel BES1/BZR1-like transcription factor that while not containing a β-amylase domain is most related to the BZR-Domain of BAM7 (bootstrap support 97%)

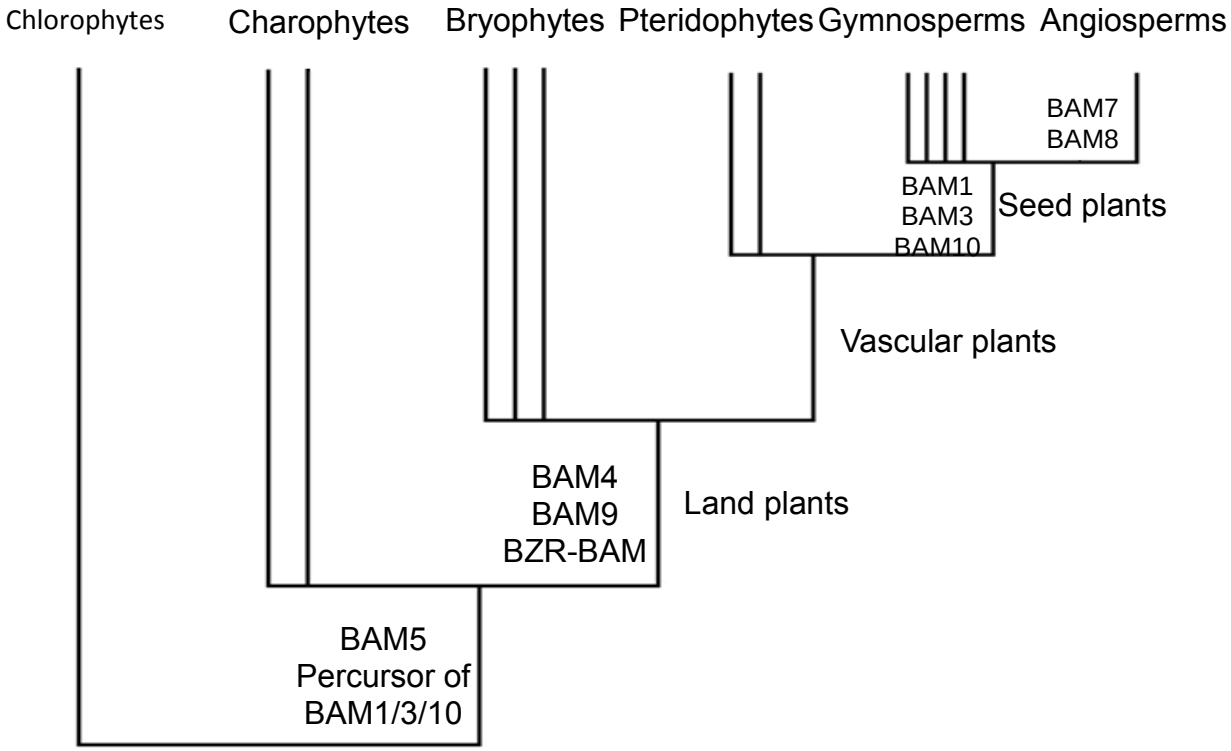


Figure 7: Estimated emergence of the different clades of β-amylases. The ancestral charophyte contained genes encoding BAM5, and an ancestral version of BAM1, BAM3 and BAM10. BAM1, BAM4, BAM9 as well as at least one gene encoding BZR-BAMs were present in the ancestor of all land plants. BAM3 and BAM10 were only found in seed plants, while BAM7 and BAM8, to BZR-BAMs are only found in flowering plants

Botanical name	Common name	Genome/ Transcriptome	website
Abies lasiocarpa	Rocky Mountain fir	Transcriptome	oneKP
Agathis robusta	Queensland kauri pine	Transcriptome	oneKP
Amborella trichopoda		Genome	NCBI blast
Angiopteris evecta	Giant fern	Transcriptome	oneKP
Aquilegia coerulea	Colorado blue Columbine	Genome	Phytozome
Arabidopsis lyrata		Genome	NCBI blast
Arabidopsis thaliana	Thale cress	Genome	NCBI blast
Arachis duranensis		Genome	http://www.peanutbase.org/
Arachis ipaensis		Genome	http://www.peanutbase.org/
Atrichum angustatum		Transcriptome	oneKP
Beta vulgaris	Sugar beet	Genome	NCBI blast
Brachypodium distachyon	Purple false brome	Genome	NCBI blast
Brassica napus	Rapeseed	Genome	NCBI blast
Brassica rapa	Field mustard	Genome	NCBI blast
Camelina sativa	False flax	Genome	NCBI blast
Camellia sinensis	Tea plant	Genome	NCBI blast
Capsella rubella	Pink shepherd's purse	Genome	NCBI blast
Capsicium annuum	Chilli	Genome	http://peppersequence.genomics.cn/page/species/index.jsp
Carica papaya	Papaya	Genome	Phytozome
Cathaya argyrophylla		Transcriptome	oneKP
Cedrus libani	Lebanon cedar	Transcriptome	oneKP
Cicer arietinum	Chickpea	Genome	NCBI blast
Citrullus lanatus	Water melon	Genome	http://www.icugi.org
Citrus clementia	Clementine	Genome	NCBI blast
Citrus sinensis	Sweet orange	Genome	NCBI blast
Coffea canephora	Coffee	Genome	NCBI blast
Coleochaete irregularis		Transcriptome	oneKP
Coleochaete scutata		Transcriptome	oneKP
Cucumis melo	Muskmelon	Genome	NCBI blast
Cucumis sativus	Cucumber	Genome	NCBI blast
Culcita macrocarpa		Transcriptome	oneKP
Cupressus dupreziana	Moroccan cypress	Transcriptome	oneKP
Cycas micholitzii		Transcriptome	oneKP
Cynara cardunculus	Artichoke	Genome	NCBI blast
Daucus carota	Carrot	Genome	NCBI blast
Dion edule	Chestnut Dion	Transcriptome	oneKP
Elaeis guineesis	African oil palm	Genome	NCBI blast
Encephalartos barteri		Transcriptome	oneKP
Ephedra sinica	Ma Huang	Transcriptome	oneKP
Equisetum diffusum	Himalayan horsetail	Transcriptome	oneKP

Equisetum hyemale	Rough horsetail	Transcriptome	oneKP
Erythranthe guttatus	Seep Monkeyflower	Genome	NCBI blast
Eucalyptus grandis	Rose gum	Genome	NCBI blast
Eutrema salsuginea	Saltwater cress	Genome	NCBI blast
Fragaria Vesca	Strawberry	Genome	NCBI blast
Ginkgo biloba	Maidenhair tree	Transcriptome	oneKP
Glycine max	Soybean	Genome	NCBI blast
Gnetum montanum		Transcriptome	oneKP
Gossypium raimondii	Cotton	Genome	NCBI blast
Griselinia racemosa		Transcriptome	oneKP
Hordeum vulgare	Barley	Genome	NCBI blast
Huperzia lucidula	Shining firmoss	Transcriptome	oneKP
Huperzia myrsinites		Transcriptome	oneKP
Ilex vomitoria	Yaupon holly	Transcriptome	oneKP
Jatropha curcas	Barbados nut	Genome	NCBI blast
Juniperus scopulorum	Rocky Mountain juniper	Transcriptome	oneKP
Keteleeria evelyniana		Transcriptome	oneKP
Leontopodium alpinum	Edelweiss	Transcriptome	oneKP
Linum usitatissimum	Common flax	Genome	Phytozome
Malus domestica	Apple	Genome	NCBI blast
Manihot esculenta	Cassava	Genome	Phytozome
Marchantia paleacea		Transcriptome	oneKP
Marchantia polymorpha	Umbrella liverwort	Transcriptome	oneKP
Medicago truncatula	Barrelclover	Genome	NCBI blast
Morus notabilis	Mulberry	Genome	NCBI blast
Musa acuminata	Banana	Genome	NCBI blast
Nageia nagi	Asian bayberry	Transcriptome	oneKP
Nelumbo nucifera	Sacred lotus	Genome	NCBI blast
Nicotiana benthamiana	Tobacco	Genome	http://sydney.edu.au/science/molecular_bioscience/sites/benthamiana/
Nothoceros aenigmaticus		Transcriptome	oneKP
Nothoceros vincentianus		Transcriptome	oneKP
Ophioglossum vulgatum	Southern adders-tongue	Transcriptome	oneKP
Oryza brachyantha		Genome	NCBI blast
Oryza sativa	Rice	Genome	NCBI blast
Panicum virgatum	Switchgrass	Genome	Phytozome
Phaseolus vulgaris	Common bean	Genome	NCBI blast
Phoenix dactylifera	Date palm	Genome	NCBI blast
Phyllostachys heterocycla	Moso bamboo	Genome	http://202.127.18.221/bamboo/down.php
Physcomitrella patens	Spreading earthmoss	Genome	NCBI blast
Picea abies	Norway spruce	Genome	http://congenie.org/start
Picea engelmandii	White spruce	Transcriptome	oneKP
Pinus radiata	Monterey pine	Transcriptome	oneKP

Pinus taeda	Loblolly pine	Genome	http://dendrome.ucdavis.edu/resources/blast/
Podocarpus coriaceus	Yucca plum pine	Transcriptome	oneKP
Podocarpus rubens		Transcriptome	oneKP
Populus trichocarpa	California poplar	Genome	NCBI blast
Prunus mume	Mei	Genome	NCBI blast
Prunus persica	Peach	Genome	NCBI blast
Pseudolarix amabilis	Golden larch	Transcriptome	oneKP
Pseudotsuga wilsoniana	Chinese Douglas fir	Transcriptome	oneKP
Psilotum nudum	Skeleton fork fern	Transcriptome	oneKP
Ricinus Communis	Castorbean	Genome	NCBI blast
Salix purpurea	Purple willow	Genome	Phytozome
Sciadoptys verticillata	Japanese umbrella-pine	Transcriptome	oneKP
Selaginella acanthonota		Transcriptome	oneKP
Selaginella kraussiana	African clubmoss	Transcriptome	oneKP
Selaginella moellendorffii		Genome	NCBI blast
Sequoiadendron giganteum	Giant sequoia	Transcriptome	oneKP
Sesamum indicum	Sesame	Genome	NCBI blast
Setaria italica	Foxtail millet	Genome	NCBI blast
Solanum lycopersicum	Tomato	Genome	NCBI blast
Solanum tuberosum	Potato	Genome	NCBI blast
Sorghum bicolor	Sorghum	Genome	NCBI blast
Spirodela polyrhiza	Common duckweed	Genome	pgir.rutgers.edu/blast/blast.cgi
Spirogyra sp	Water silk	Transcriptome	oneKP
Stangeria eriopus		Transcriptome	oneKP
Taiwania cryptomerioides		Transcriptome	oneKP
Takakia lepidozioides		Transcriptome	oneKP
Tarenaya hassleriana	Spider flower	Genome	NCBI blast
Taxus baccata	European yew	Transcriptome	oneKP
Theobroma cacao	Cacao	Genome	NCBI blast
Torreya nucifera	Japanese nutmeg-yew	Transcriptome	oneKP
Treubia lacunosa		Transcriptome	oneKP
Tsuga heterophylla	Western Hemlock	Transcriptome	oneKP
Vitis vinifera	Grape	Genome	NCBI blast
Welwitschia mirabilis		Transcriptome	oneKP
Wollemia nobilis	Wollemi pine	Transcriptome	oneKP
Zea mays	Maize	Genome	NCBI blast

Supplementary table 1: Source of the β-amylase sequences used in this study. The majority of the sequences was retrieved from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html), oneKP (https://www.bioinfodata.org/Blast4OneKP/) and NCBI blast

(https://blast.ncbi.nlm.nih.gov/Blast.cgi). Additional websites are listed in the table itself.

Name	Length	Score	cTP	CS-score	cTP-length
BAM1.1_A.robusta_MIXZ2012495	617	0.53	Y	-1.61	40
BAM1.1_B.distachyon_XP_003558837.1	573	0.583	Y	4.748	39
BAM1.1_B.napus_XP_013747707.1	570	0.57	Y	5.589	74
BAM1.1_B.rapa_XP_009102529.1	570	0.57	Y	5.589	74
BAM1.1_C.arietinum_XP_004515248.1	573	0.54	Y	12.047	31
BAM1.1_C.melo_XP_008438436.1	577	0.543	Y	2.009	49
BAM1.1_C.sativus_XP_004134029.1	577	0.527	Y	2.465	34
BAM1.1_Camelina_sativa_XP_010488521.1	575	0.548	Y	4.618	67
BAM1.1_F.vesca_XP_004296549.1	578	0.547	Y	9.41	34
BAM1.1_G.max_XP_003534086.1	569	0.521	Y	4.776	69
BAM1.1_G.raimondii_XP_012454771.1	587	0.509	Y	3.219	29
BAM1.1_H.vulgare_BAJ96121.1	551	0.54	Y	6.994	30
BAM1.1_M.domestica_XP_008391283.1	571	0.549	Y	6.728	80
BAM1.1_M.esculenta_cassava4.1_004325m	582	0.531	Y	13.835	34
BAM1.1_N.benthamiana_Nbv5tr6236522	576	0.516	Y	5.069	80
BAM1.1_N.nucifera_XP_010263970.1	568	0.536	Y	4.346	71
BAM1.1_O.sativa_NP_001048926.1	557	0.542	Y	1.751	38
BAM1.1_P.coriaceus_SCEB2055235	620	0.519	Y	5.627	13
BAM1.1_P.dactylifera_XP_008775132.1	572	0.451	-	2.793	69
BAM1.1_P.engelmandii_AWQB200193	623	0.478	-	2.195	76
BAM1.1_P.rubens_BAM1.1_XLGK2007963	621	0.528	Y	5.627	13
BAM1.1_P.taeda_PITA_000025218RA	623	0.451	-	1.035	13
BAM1.1_P.trichocarpa_XP_002311706.1	562	0.506	Y	5.628	62
BAM1.1_P.vulgaris_XP_007152599.1	568	0.54	Y	5.977	68
BAM1.1_S.bicolor_XP_002468533.1	564	0.565	Y	3.825	55
BAM1.1_S.indicum_XP_011091372.1	580	0.547	Y	2.879	70
BAM1.1_S.italica_XP_004985750.1	563	0.563	Y	5.856	79
BAM1.1_S.lycopersicum_NP_001234556.2	580	0.538	Y	5.069	84
BAM1.1_S.purpurea_SapurV1A.0021s0270	582	0.519	Y	12.613	30
BAM1.1_S.tuberosum_XP_006340896.1	579	0.541	Y	3.915	85
BAM1.1_T.heterophylla_GAMH2004527	611	0.447	-	0.85	64
BAM1.1_V.vinifera_XP_002285569.1	573	0.491	-	11.114	34
BAM1.1_Z.mays_NP_001147532.1	544	0.58	Y	10.628	63
BAM1.2_B.distachyon_XP_003571854.1	534	0.588	Y	13.063	59
BAM1.2_B.napus_XP_013750064.1	564	0.576	Y	5.589	68
BAM1.2_B.rapa_XP_009135884.1	564	0.543	Y	5.589	70
BAM1.2_C.melo_XP_008460711.1	545	0.549	Y	7.246	72
BAM1.2_C.sativus_XP_004147196.1	545	0.563	Y	7.246	72
BAM1.2_G.max_XP_003548316.1	575	0.548	Y	4.624	75
BAM1.2_M.domestica_XP_008342553.1	571	0.551	Y	5.208	80

BAM1.2_M.esculenta_cassava4.1_004345m	581	0.522	Y	6.559	75
BAM1.2_N.benthamiana_Nbv5tr6214720	565	0.536	Y	4.153	48
BAM1.2_O.sativa_NP_001064798.1	535	0.581	Y	9.594	62
BAM1.2_P.heterocycla_PH01000560G0630	449	0.483	-	5.868	31
BAM1.2_P.taeda_PITA_000025216RA	620	0.463	-	2.333	77
BAM1.2_P.trichocarpa_XP_002314522.2	586	0.465	-	4.905	45
BAM1.2_S.bicolor_XP_002467119.1	547	0.584	Y	7.393	64
BAM1.2_S.indicum_XP_011073736.1	583	0.558	Y	3.163	82
BAM1.2_S.purpurea_SapurV1A.0096s0290	584	0.461	-	5.628	82
BAM1.2_Z.mays_NP_001148159.1	573	0.556	Y	10.374	26
BAM1.3_Camelina_sativa_XP_010513371.1	576	0.554	Y	4.624	75
BAM1.3_G.raimondii_XP_012484484.1	589	0.534	Y	2.763	66
BAM1.3_N.nagi_UUJS2117213	620	0.507	Y	5.627	13
BAM1.3_N.nucifera_XP_010264799.	594	0.5	-	13.774	33
BAM1.3_P.heterocycla_PH01000010G113	484	0.48	-	2.921	44
BAM1.3_P.taeda_PITA_000025217RA	623	0.446	-	7.149	69
BAM1.4_P.taeda_PITA_000025219RA	580	0.528	Y	7.031	46
BAM1_A.corulea_Aquca_025_00081	595	0.535	Y	5.186	36
BAM1_A.ipaensis_Araip.183TE	519	0.555	Y	2.992	68
BAM1_A.lasiocarpa_VSRH2002636	622	0.456	-	2.195	76
BAM1_A.lyrata_XP_002885629.1	572	0.53	Y	5.661	40
BAM1_A.thaliana_NP_189034.1	575	0.524	Y	5.661	41
BAM1_A.trichopoda_XP_006851336.1	587	0.541	Y	8.354	36
BAM1_B.vulgaris_XP_010666969.1	579	0.528	Y	6.763	101
BAM1_C.annum_Capana03g004414	577	0.516	Y	4.86	41
BAM1_C.argyrophylla_NPRL2008412	622	0.451	-	6.203	101
BAM1_C.canephora_CDP20299.1	582	0.525	Y	10.925	73
BAM1_C.cardunculus_KVH91041.1	572	0.54	Y	7.816	65
BAM1_C.clementia_XP_006420416.1	580	0.494	-	7.65	90
BAM1_C.lantus_Cla007635	554	0.571	Y	11.184	73
BAM1_C.papaya_evm.TU.supercontig_59.40	574	0.548	Y	6.253	33
BAM1_C.rubella_XP_006296695.1	573	0.535	Y	5	71
BAM1_Camellia.sinensis_AKQ62956.1	581	0.538	Y	5.895	47
BAM1_Citrus.sinensis_XP_006493994.1	580	0.498	-	7.65	90
BAM1_D.carota_KZM87169.1	569	0.504	Y	6.861	45
BAM1_E.grandis_XP_010023784.1	584	0.492	-	13.835	34
BAM1_E.guineesis_XP_010918964.1	571	0.454	-	10.873	29
BAM1_E.guttatus_XP_012829096.1	574	0.563	Y	6.906	98
BAM1_E.salsuginea_XP_006418770.1	582	0.539	Y	7.111	79
BAM1_G.racemosa_QIKZ2005472	454	0.49	-	7.816	70
BAM1_I.vomitoria_ASMV2109428	577	0.505	Y	5.069	79
BAM1_J.curcas_XP_012077650.1	583	0.489	-	6.203	75
BAM1_L.alpinum_DOVJ2001331	572	0.511	Y	7.816	70
BAM1_M.acuminata_XP_009400488.1	590	0.522	Y	4.175	79
BAM1_M.notabilis_XP_010111574.1	604	0.553	Y	0.627	84
BAM1_M.trunculata_XP_013452815.1	572	0.51	Y	4.772	44

BAM1_O.brachyantha_XP_006662424.1	453	0.451	-	1.567	60
BAM1_P.abies_MA_124514g0010	624	0.479	-	4.012	55
BAM1_P.mume_XP_008224054.1	569	0.555	Y	4.173	77
BAM1_R.communis_XP_002518196.1	574	0.494	-	7.963	78
BAM1_S.polyrhiza_Spipo16G0046000	575	0.453	-	3.779	81
BAM1_T.hassleriana_XP_010550849.1	591	0.513	Y	13.302	83

Supplementary table 2: Predicted localisation of BAM1 orthologs

Name	Length	Score	cTP	CS-score	cTP-length
BAM2.1_B.napus_XP_013660393.1	533	0.532	Y	6.354	23
BAM2.1_Camelina_sativa_XP_010427089.1	554	0.559	Y	1.929	51
BAM2.1_P.dactylifera_XP_008787503.1	534	0.566	Y	2.459	57
BAM2_V.vinifera_XP_002274612.2	554	0.583	Y	3.939	61
BAM2.2_B.napus_XP_013731270.1	539	0.526	Y	6.215	48
BAM2.2_Camelina_sativa_XP_010456279.1	555	0.564	Y	1.929	50
BAM2.2_P.dactylifera_XP_008804014.1	548	0.552	Y	3.861	62
BAM2_A.lyrata_XP_002875024.1	542	0.563	Y	5.840	56
BAM2_A.thaliana_NP_191958.3	542	0.557	Y	5.840	55
BAM2_B.distachyon_XP_010232924.1	530	0.432	-	2.404	89
BAM2_B.rapa_XP_009111477.1	541	0.53	Y	3.035	48
BAM2_B.vulgaris_XP_010670423.1	574	0.578	Y	4.919	55
BAM2_C.annum_Capana08g000914	433	0.449	-	5.558	16
BAM2_C.clementia_XP_006445046.1	562	0.555	Y	-0.819	66
BAM2_C.papaya_evm.TU.supercontig_18.253	504	0.574	Y	1.152	45
BAM2_C.rubella_XP_006287426.1	549	0.553	Y	5.533	49
BAM2_Camellia.sinensis_AHC32020.1	556	0.575	Y	2.422	60
BAM2_Citrus.sinensis_XP_006491095.1	562	0.555	Y	-0.819	66
BAM2_E.grandis_XP_010055131.1	546	0.561	Y	9.724	67
BAM2_E.guineesis_XP_010934793.1	553	0.58	Y	1.456	46
BAM2_E.guttatus_XP_012843727.1	558	0.538	Y	4.775	55
BAM2_E.salsuginea_XP_006396247.1	546	0.552	Y	1.929	52
BAM2_F.vesca_XP_004306786.1	544	0.538	Y	1.217	10
BAM2_G.raimondii_XP_012489942.1	536	0.54	Y	6.041	55
BAM2_J.curcas_XP_012083395.1	537	0.548	Y	-0.221	46
BAM2_M.acuminata_XP_009392820.1	541	0.523	Y	2.448	21
BAM2_M.domestica_XP_008338858.1	548	0.562	Y	4.418	74
BAM2_M.esculenta_cassava4.1_022883m	540	0.576	Y	6.740	58
BAM2_M.notabilis_XP_010105936.1	554	0.586	Y	5.457	36
BAM2_O.sativa_NP_001063976.1	533	0.573	Y	0.678	40
BAM2_P.mume_XP_008232901.1	538	0.582	Y	8.237	71
BAM2_P.trichocarpa_XP_002320794.2	539	0.569	Y	3.519	37
BAM2_R.communis_XP_0	609	0.576	Y	7.687	45
BAM2_S.indicum_XP_01	527	0.542	Y	8.744	52
BAM2_S.italica_XP_012699441.1	566	0.548	Y	2.474	17
BAM2_T.cacao_XP_007051810.1	554	0.568	Y	2.583	48

BAM2_T.hassleriana_XP_010540283.1	550	0.512	Y	3.043	20

Supplementary table 3: Predicted localisation of BAM2 orthologs

Name	Length	Score	cTP	CS-score	cTP-length
BAM3.1_B.napus_XP_013721634.1	549	0.515	Y	1.424	48
BAM3.1_B.rapa_XP_009136825.1	548	0.51	Y	1.424	48
BAM3.1_C.arietinum_XP_004511752.1	545	0.447	-	1.775	63
BAM3.1_Camelina_sativa_XP_010440058.1	548	0.519	Y	1.424	49
BAM3.1_E.guttatus_XP_012829112.1	553	0.512	Y	3.481	42
BAM3.1_G.max_XP_006573703.1	548	0.5	Y	3.437	43
BAM3.1_G.raimondii_XP_012488822.1	539	0.492	-	1.138	54
BAM3.1_M.acuminata_XP_009397011.1	547	0.526	Y	9.827	44
BAM3.1_M.trunculata_XP_003611408.1	543	0.444	-	-0.289	32
BAM3.1_P.abies_MA_129283g0010	552	0.525	Y	-1.768	49
BAM3.1_P.taeda_PITA_000032112RA	557	0.525	Y	4.899	2
BAM3.1_P.trichocarpa_XP_006385389.1	547	0.495	-	5.345	40
BAM3.1_S.lycopersicum_XP_004244551.1	542	0.45	-	-1.196	13
BAM3.1_S.purpurea_SapurV1A.0380s0080	547	0.513	Y	7.486	40
BAM3.1_S.tuberosum_XP_006362484.1	541	0.466	-	5.338	39
BAM3.1_Z.mays_XP_008658990.1	553	0.519	Y	9.935	48
BAM3.2_B.napus_XP_013737190.1	548	0.51	Y	1.424	48
BAM3.2_B.rapa_XP_009144721.1	549	0.515	Y	1.424	48
BAM3.2_C.arietinum_XP_004508980.1	545	0.468	-	5.353	58
BAM3.2_Camelina_sativa_XP_010449668.1	548	0.524	Y	1.424	49
BAM3.2_D.carota_KZN07474.1	545	0.53	Y	5.120	31
BAM3.2_E.guttatus_XP_012852342.1	553	0.517	Y	3.481	42
BAM3.2_G.max_NP_001236350.1	540	0.529	Y	3.295	45
BAM3.2_G.raimondii_XP_012439399.1	508	0.513	Y	4.235	42
BAM3.2_M.acuminata_XP_009413253.1	549	0.485	-	-1.056	45
BAM3.2_M.trunculata_XP_003611409.1	543	0.473	-	2.905	63
BAM3.3_M.trunculata_XP_013457558.1	541	0.47	-	3.687	54
BAM3.2_P.abies_MA_3193g0010	552	0.507	Y	-0.356	49
BAM3.2_P.taeda_PITA_000045784RA	557	0.511	Y	2.237	53
BAM3.2_P.trichocarpa_XP_006385589.1	548	0.512	Y	5.345	40
BAM3.2_S.lycopersicum_XP_004245844.1	546	0.497	-	5.289	41
BAM3.2_S.purpurea_SapurV1A.0241s0110	552	0.481	-	2.917	32
BAM3.2_S.tuberosum_NP_001275172.1	545	0.477	-	5.289	40
BAM3.2_Z.mays_XP_008658465.1	553	0.519	Y	9.935	48
BAM3.3_Camelina_sativa_XP_010434720.1	548	0.519	Y	1.424	49
BAM3.3_G.max_XP_003539125.1	554	0.49	-	3.437	43
BAM3.3_P.taeda_PITA_000047084RA	593	0.497	-	0.683	23
BAM3.4_G.max_XP_003524296.1	547	0.503	Y	3.920	40
BAM3_A.corulea_Aquca_053_00137	548	0.538	Y	1.306	40
BAM3_A.ipaensis_Araip.67F6M	564	0.487	-	2.325	64
BAM3_A.lyrata_XP_002868085.1	548	0.521	Y	1.424	49

BAM3_A.thaliana_NP_567523.1	548	0.518	Y	1.424	49
BAM3_A.trichopoda_XP_011621487.1	552	0.478	-	2.384	43
BAM3_B.distachyon_XP_003574353.1	548	0.505	Y	7.257	45
BAM3_B.vulgaris_XP_010695452.1	546	0.452	-	6.100	16
BAM3_C.annum_Capana01g000793	545	0.509	Y	5.289	40
BAM3_C.canephora_CDP13430.1	547	0.531	Y	1.862	42
BAM3_C.cardunculus_KVH95266.1	556	0.487	-	5.009	56
BAM3_C.clementia_XP_006440139.1	551	0.522	Y	5.478	66
BAM3_C.lantus_Cla008766	537	0.449	-	2.048	46
BAM3_C.melo_XP_008448759.1	537	0.439	-	2.048	46
BAM3_C.rubella_XP_006285145.1	548	0.527	Y	1.424	49
BAM3_C.sativus_XP_004147264.1	538	0.442	-	2.048	46
BAM3_Camellia.sinensis_AHJ09602.1	548	0.53	Y	2.756	41
BAM3_Citrus.sinensis_XP_006477060.1	551	0.522	Y	5.478	66
BAM3_E.grandis_XP_010055392.1	543	0.494	-	2.550	42
BAM3_E.guineesis_XP_010919815.1	546	0.464	-	0.367	23
BAM3_E.salsuginea_XP_006414272.1	548	0.526	Y	1.424	48
BAM3_F.vesca_XP_004300297.1	553	0.516	Y	5.120	34
BAM3_G.racemosa_QIKZ2022040	538	0.471	-	1.630	34
BAM3_H.vulgare_BAJ90222.1	549	0.496	-	7.257	47
BAM3_I.vomitoria_ASMV2109425	542	0.502	Y	1.516	40
BAM3_J.curcas_XP_012075356.1	547	0.488	-	0.606	32
BAM3_L.alpinum_DOVJ2007219	468	0.446	-	-5.321	2
BAM3_M.domestica_XP_008361217.1	547	0.477	-	4.715	40
BAM3_M.esculenta_cassava4.1_034006m	572	0.476	-	3.151	42
BAM3_M.notabilis_XP_010110537.1	544	0.472	-	5.707	45
BAM3_N.benthamiana_Nbv5tr6221381	548	0.459	-	2.349	42
BAM3_N.nucifera_XP_010274550.1	547	0.513	Y	2.670	43
BAM3_O.brachyantha_XP_006662612.1	305	0.506	Y	3.821	53
BAM3_O.sativa_NP_001065418.2	522	0.513	Y	6.307	45
BAM3_P.dactylifera_XP_008794866.1	547	0.507	Y	8.529	46
BAM3_P.mume_XP_008229498.1	547	0.461	-	4.715	40
BAM3_P.persica_XP_007209867.1	547	0.481	-	4.715	40
BAM3_P.vulgaris_XP_007155732.1	548	0.46	-	4.468	63
BAM3_R.communis_XP_002517513.1	547	0.497	-	3.878	40
BAM3_S.bicolor_XP_002464915.1	557	0.509	Y	8.636	53
BAM3_S.indicum_XP_011070282.1	549	0.519	Y	5.168	38
BAM3_S.italica_XP_004983616.1	557	0.527	Y	13.487	54
BAM3_T.cacao_XP_007039629.1	575	0.576	Y	2.962	59
BAM3_T.hassleriana_XP_010531694.1	549	0.5	-	3.595	44
BAM3_V.vinifera_XP_002282871.1	543	0.509	Y	3.869	41

Supplementary table 4: Predicted localisation of BAM3 orthologs

Name	Length	Score	cTP	CS-score	cTP-length
BAM4.1_B.napus_XP_013685544.1	523	0.57	Y	3.653	78

BAM4.1_B.rapa_XP_009132357.1	532	0.532	Y	0.433	61
BAM4.1_Camelina_sativa_XP_010483031.1	531	0.533	Y	2.467	86
BAM4.2_B.napus_XP_013676174.1	528	0.529	Y	3.653	83
BAM4.2_B.rapa_XP_009126986.1	528	0.516	Y	3.653	83
BAM4.2_Camelina_sativa_XP_010450090.1	531	0.542	Y	2.467	86
BAM4_A.corulea_Aquca_005_00321	528	0.55	Y	3.932	17
BAM4_A.lyrata_XP_002864407.1	531	0.539	Y	2.467	86
BAM4_A.thaliana_NP_568829.2	531	0.541	Y	2.600	62
BAM4_B.vulgaris_XP_010675936.1	524	0.472	-	0.385	76
BAM4_C.cardunculus_KVH99190.1	574	0.482	-	4.998	57
BAM4_C.lantus_Cla004462	527	0.526	Y	1.593	57
BAM4_C.melo_XP_008449033.1	524	0.51	Y	1.656	62
BAM4_C.papaya_evm.model.supercontig_12.148	431	0.541	Y	-0.993	72
BAM4_C.rubella_XP_006280293.1	531	0.535	Y	2.600	62
BAM4_C.sativus_XP_004148285.1	520	0.56	Y	1.965	61
BAM4_Camellia.sinensis_AKQ62957.1	518	0.461	-	5.086	72
BAM4_E.salsuginea_XP_006401422.1	531	0.526	Y	-2.824	33
BAM4_F.vesca_XP_004291809.1	516	0.551	Y	4.784	80
BAM4_G.raimondii_XP_012473201.1	518	0.534	Y	1.593	58
BAM4_J.curcas_XP_012071010.1	521	0.495	-	2.054	75
BAM4_L.alpinum_DOVJ2015003	495	0.533	Y	5.932	40
BAM4_M.domestica_XP_008366443.1	525	0.515	Y	3.843	12
BAM4_M.esculenta_cassava4.1_005532m	522	0.535	Y	-1.003	14
BAM4_N.nucifera_XP_010270251.1	447	0.465	-	2.873	73
BAM4_P.mume_XP_008227162.1	521	0.479	-	6.111	10
BAM4_P.taeda_PITA_000022542RA	574	0.531	Y	2.178	96
BAM4_T.cacao_XP_007020502.1	521	0.469	-	1.593	61
BAM4_T.hassleriana_XP_010536726.1	531	0.537	Y	-0.249	62
BAM4_V.vinifera_XP_002265698.1	522	0.452	-	1.472	85

Supplementary table 5: Predicted localisation of BAM4 orthologs

Name	Length	Score	cTP	CS-score	cTP-length
BAM5.1_B.napus_XP_013655702.1	498	0.433	-	1.783	26
BAM5.1_B.rapa_XP_009107836.1	498	0.433	-	1.783	26
BAM5.1_Camelina_sativa_XP_010435035.1	498	0.431	-	1.783	26
BAM5.1_Z.mays_NP_001105496.1	488	0.434	-	4.366	34
BAM5.2_B.napus_XP_013653901.1	520	0.427	-	-4.577	23
BAM5.2_B.rapa_XP_009106879.1	498	0.427	-	-4.577	23
BAM5.2_Camelina_sativa_XP_010440324.1	498	0.433	-	1.783	26
BAM5.2_Z.mays_NP_001168436.1	595	0.572		4.627	49
BAM5.3_Camelina_sativa_XP_010449959.1	498	0.433	-	1.783	26
BAM5.1_B.vulgaris_XP_010688831.1	571	0.515	Y	0.191	47
BAM5.1_C.annum_Capana07g001521	572	0.501	Y	3.879	64
BAM5.1_C.arietinum_XP_004487367.1	595	0.438	-	-0.926	5
BAM5.1_E.guttatus_XP_012852439.1	584	0.53	Y	3.078	70

BAM6.1_G.max_XP_003539882.1	496	0.438	-	1.195	25
BAM5.1_G.raimondii_XP_012434830.1	492	0.431	-	2.403	8
BAM5.1_H.vulgare_BAA04815	535	0.436	-	3.357	6
BAM5.1_M.acuminata_XP_009388866.1	503	0.441	-	5.838	27
BAM5.1_M.trunculata_XP_013455524.1	496	0.436	-	0.482	25
BAM5.1_O.brachyantha_XP_006657803.1	488	0.438	-	3.903	6
BAM5.1_P.heterocycla_PH01000656G0560	488	0.433	-	5.951	34
BAM5.1_S.italica_XP_004957938.1	488	0.432	-	4.366	34
BAM5.1_T.cacao_XP_007029518.1	500	0.432	-	2.071	2
BAM5.2_A.ipaensis_Araip.YCB0N	611	0.435	-	3.826	53
BAM5.2_B.vulgaris_XP_010689279.1	492	0.433	-	2.505	12
BAM5.2_C.arietinum_XP_004513548.1	496	0.446	-	3.365	25
BAM5.2_E.guttatus_XP_012852440.1	456	0.531	Y	1.283	24
BAM5.2_G.max_XP_003540325.2	601	0.442	-	4.941	73
BAM5.2_G.raimondii_XP_012479806.1	600	0.446	-	3.396	6
BAM5.2_H.vulgare_AAX37358.1	505	0.434	-	3.815	6
BAM5.2_M.acuminata_XP_009420599.1	505	0.427	-	6.204	5
BAM5.2_M.trunculata_XP_003597045.2	590	0.465	-	3.339	45
BAM5/6.2_N.nucifera_XP_010267811.1	519	0.44	-	1.914	14
BAM5.2_O.brachyantha_XP_006658660.1	478	0.564	Y	6.592	64
BAM5.2_O.sativa_NP_001059906.1	600	0.567	Y	7.392	58
BAM5.2_P.heterocycla_PH01002417G0300	488	0.438	-	3.815	6
BAM5.2_S.italica_XP_004957937.1	587	0.57	Y	6.352	35
BAM5.2_T.cacao_XP_007043355.1	410	0.443	-	4.354	68
BAM5.3_B.vulgaris_XP_010695752.1	492	0.436	-	2.505	12
BAM5.3_G.max_XP_003541934.2	592	0.441	-	1.594	38
BAM5.3_H.vulgare_BAK00030.1	603	0.548	Y	5.226	67
BAM5.3_P.heterocycla_PH01002346G0350	602	0.541	Y	6.878	30
BAM5_A.corulea_Aquca_005_00422	589	0.452	-	9.552	60
BAM5_A.duranensis_Aradu.A6XWX.1	527	0.436	-	2.403	14
BAM5_C.canephora_CDP20214.1	491	0.455	-	1.577	22
BAM5_C.clementia_XP_006447463.1	519	0.431	-	-1.856	2
BAM5_C.lantus_Cla007699	578	0.47	-	6.823	11
BAM5_C.macrocarpa_PNZO2024341	497	0.43	-	-2.009	18
BAM5_C.melo_XP_008455397.1	583	0.464	-	1.017	68
BAM5_C.sativus_XP_011658712.1	583	0.449	-	0.007	70
BAM5_C.sinensis_AHG94609.1	593	0.448	-	3.132	20
BAM5_E.grandis_XP_010051211.1	531	0.429	-	-0.596	81
BAM5_M.domestica_XP_008379598.1	598	0.513	Y	4.116	36
BAM5_M.notabilis_XP_010109553.1	511	0.434	-	-0.283	2
BAM5_N.benthamiana_Nbv5tr6213155	614	0.499	-	4.103	67
BAM5_P.mume_XP_008239169.1	515	0.432	-	-3.304	6
BAM5_P.persica_XP_007215122.1	516	0.436	-	-2.962	6
BAM5_P.radiata_DZQM2008648	478	0.433	-	0.568	33
BAM5_P.taeda_PITA_000048102RA	549	0.425	-	-2.337	19
BAM5_P.vulgaris_XP_007149944.1	587	0.44	-	2.451	26

BAM5_S.bicolor_XP_002460819.1	604	0.57	Y	4.627	51
BAM5_S.polyrhiza_Spipo7G0011700	401	0.487	-	7.017	15
BAM5_S.purpurea_SapurV1A.0312s0210	582	0.449	-	8.030	55
BAM5_A.lyrata_XP_002868220.1	499	0.433	-	1.136	26
BAM5_A.thaliana_NP_567460.1	420	0.435	-	1.783	26
BAM5_A.trichopoda_XP_006837006.2	717	0.479	-	5.505	49
BAM5_E.guineesis_XP_010931493.1	519	0.44	-	5.511	30
BAM5_E.salsuginea_XP_006414555.1	498	0.436	-	1.783	26
BAM5_F.vesca_XP_004289151.1	586	0.508	Y	1.288	31
BAM5_J.curcas_XP_012086395.1	518	0.433	-	3.676	28
BAM5_P.dactylifera_XP_008793743.1	524	0.439	-	3.727	29
BAM5_P.vulgaris_XP_007132589.1	497	0.443	-	4.942	45
BAM5_R.communis_XP_002515712.1	518	0.431	-	5.284	28
BAM5_S.indicum_XP_011100422.1	579	0.479	-	5.368	61
BAM5_S.lycopersicum_XP_004243448.1	575	0.487	-	3.975	61
BAM5_S.tuberosum_XP_006360578.1	578	0.508	Y	3.789	64
BAM5_V.vinifera_XP_002281003.2	596	0.513	Y	2.413	13
BAM5_C.sinensis_XP_006469732.1	519	0.431	-	-1.856	2
BAM5_P.trichocarpa_XP_006372990.1	583	0.434	-	-1.076	13

Supplementary table 6: Predicted localisation of BAM5 orthologs

Name	Length	Score	cTP	CS-score	cTP-length
BAM6_A.thaliana_NP_180788.2	577	0.464	-	3.771	52
BAM6_A.lyrata_XP_002881219.1	577	0.457	-	0.162	5
BAM6.1_Camelina_sativa_XP_010510054.1	577	0.516	Y	4.536	49
BAM6.2_Camelina_sativa_XP_010414016.1	570	0.534	Y	4.861	45
BAM6.3_Camelina_sativa_XP_010469618.1	577	0.509	Y	2.629	50
BAM6_C.rubella_XP_006293904.1	576	0.492	-	2.629	49
BAM6_E.salsuginea_XP_006410362.1	587	0.513	Y	8.671	27
BAM6_B.rapa_XP_009143965.1	581	0.534	Y	4.371	51
BAM6.1_B.napus_XP_013748682.1	581	0.529	Y	4.371	51
BAM6.2_B.napus_XP_013675826.1	582	0.492	-	4.727	52
BAM6_T.hassleriana_XP_010522319.1	592	0.51	Y	6.190	59

Supplementary table 7: Predicted localisation of BAM6 orthologs

Name	NLS (0.6 cutoff)	NLS-sequence
BAM7.1_B.napus_XP_009143179.1	Y	63 - RRSRPVEEKERTKLRERHRRAI - 84
BAM7.1_Camelina_sativa_XP_010508098.1	Y	66 - SRRSRPLEEKERTKLRERHRRAIT - 89
BAM7.1_G.max_XP_003534564.1	Y	83 - RRSRPLEEKERTKLRERRRRAITA - 106
BAM7.1_G.raimondii_XP_012475200.1	Y	79 - ARRSRPLEEKERTKLRERHRRAI - 101
BAM7.1_N.nucifera_XP_010241901.1	Y	77 - RPKEEKERTKLRERHRRS - 94

BAM7.1_P.heterocycla_PH01001239G0650	Y	51 - RRSRAREEKERTKLRERQRRAI - 72
BAM7.1_P.trichocarpa_XP_002320793.2	Y	65 - ERTKLRERHRR - 75
BAM7.2_B.napus_XP_013748222.1	Y	63 - RRSRPVEEKERTKLRERHRRAI - 84
BAM7.2_Camelina_sativa_XP_010506568.1	Y	66 - SRRSRPLEEKERTKLRERHRRAIT - 89
BAM7.2_D.carota_KZM88115.1	-	-
BAM7.2_G.max_XP_003552392.1	Y	83 - RRSRPLEEKERTKLRERRRRAITA - 106
BAM7.2_N.nucifera_XP_010255372.1	Y	74 - PRRCRPKEEKERTKLRERHRRAIT - 97
BAM7.2_P.heterocycla_PH01002350G0210	Y	60 - GGRRSRAREEKERTKLRERQRRA - 82
BAM7.2_P.trichocarpa_XP_002302585.2	Y	77 - ARRSRPLEEKERTKLRERHRRAI - 99
BAM7.2_S.purpurea_SapurV1A.0046s0400	Y	76 - RRSRPLEEKERTKLRERHRRAI - 97
BAM7.3_Camelina_sativa_XP_010518231.1	Y	66 - SRRSRPLEEKERTKLRERHRRAIT - 89
BAM7_A.corulea_Aquca_013_00313	Y	94 - RRCRPREEKERTKLRERHRRAI - 115
BAM7_A.duranensis_Aradu.R0WSE.1	Y	37 - KERTKLRERRRRAITA - 52
BAM7_A.ipaensis_Araip.5ZJ5X.1	Y	90 - KERTKLRERRRRAITA - 105
BAM7_A.lyrata_XP_002882038.1	Y	66 - SRRSRPLEEKERTKLRERHRRAIT - 89
BAM7_A.thaliana_NP_182112.2	Y	67 - SRRSRPLEEKERTKLRERHRRAIT - 90
BAM7_A.trichopoda_XP_006827627.2	Y	70 - RRRCRPKEEKERTKLRERHRRAIT - 93
BAM7_B.rapa_XP_009143179.1	Y	63 - RRSRPVEEKERTKLRERHRRAI - 84
BAM7_B.vulgaris_XP_010670436.1	Y	76 - RRSRPLEEKERTKLRERHRRAITARILAGLRR
BAM7_C.annum_Capana08g000917	-	-
BAM7_C.canephora_CDP08819.1	-	-
BAM7_C.cardunculus_KVH89110.1	-	-
BAM7_C.clementia_XP_006445048.1	-	-
BAM7_C.lantus_Cla001224	Y	84 - KERTKLRERHRRA - 96
BAM7_C.melo_XP_008458240.1	Y	84 - KERTKLRERHRRA - 96
BAM7_C.papaya_evm.model.supercontig_18.252	Y	88 - KERTKLRERHRRA - 100
BAM7_C.sativus_XP_011656338.1	Y	84 - KERTKLRERHRRA - 96
BAM7_C.sinensis_AKQ62959.1	Y	83 - EKERKKIRERQRRAV - 97
BAM7_E.grandis_XP_010055132.1	Y	75 - GARRSRPLEEKERTKLRERHRRAI - 98
BAM7_E.guineesis_XP_010909265.1	Y	83 - RRPRAKEEKERTKMRERHRRAI - 104
BAM7_E.guttatus_XP_012840197.1	-	-
BAM7_E.salsuginea_XP_006397762.1	Y	66 - SRRSRPLEEKERTKLRERHRRAIT - 89
BAM7_F.vesca_XP_004306787.1	Y	92 - TKLRERQRR - 100
BAM7_G.racemosa_QIKZ2024994	Y	89 - RER - 91
BAM7_H.vulgare_BAJ96466.1	Y	40 - RPPERRRGRGREEKERTKARERRRRAVTGRILAG - 77
BAM7_I.vomitoria_ASMV2024789	-	-
BAM7_J.curcas_XP_012083397.1	Y	77 - GARRSRPLEEKERTKLRERHRRAI - 100
BAM7_M.acuminata_XP_009398621.1	Y	80 - SRRSRPAEEKERTKLRERHRRAITG - 104
BAM7_M.domestica_XP_008338860.1	Y	93 - RTKLRERQR - 101
BAM7_M.esculenta_cassava4.1_002728m	Y	78 - ARRSRPLEEKERTKLRERHRRAI - 100
BAM7_M.notabilis_XP_010105937.1	Y	90 - KERTKLRERHRRA - 102
BAM7_M.trunculata_XP_013449334.1	Y	80 - NRRSRPVEEKERTKLRERRRRAITA - 104
BAM7_N.benthamiana_Nbv5tr6219619	Y	85 - RER - 87
BAM7_P.dactylifera_XP_008777296.1	Y	83 - RRPRPKEEKERTKMRERHRRAIT - 105
BAM7_P.mume_XP_008232902.1	-	-

BAM7_P.persica_XP_007220223.1	-	-
BAM7_P.vulgaris_XP_007139874.1	Y	81 - RRSRPVEEKERTKLRERRRRAITA - 104
BAM7_R.communis_XP_002511857.1	Y	83 - RSRPLEEKERTKLRERHRRAI - 103
BAM7_S.indicum_XP_011093139.1	Y	97 - KLRERQR - 103
BAM7_S.lycopersicum_XP_004229887.1	Y	86 - RERQ - 89
BAM7_S.polyrhiza_Spipo1G0030700	Y	99 - RCRPKEEKERTKLRERHRRAI - 119
BAM7_S.tuberosum_XP_006339564.1	Y	85 - LRERQR - 90
BAM7_T.cacao_XP_007051814.1	Y	78 - ARRSRPLEEKERTKLRERHRRAI - 100
BAM7_T.hassleriana_XP_010523740.1	Y	66 - SRRSRPLEEKERTKLRERHRRAIT - 89

Supplementary table 8: Predicted localisation of BAM7 orthologs

Name	NLS (0.6 cutoff)	NLS-sequence
BAM8.1_B.napus_XP_013721726.1	Y	73 - GGGGGKGKREREKEKERTKLRERHRRA - 99
BAM8.1_Camelina_sativa_XP_010441757.1	Y	75 - GERGKGKREREKEKERTKLRERHRRA - 100
BAM8.2_B.napus_XP_013656407.1	Y	72 - GGGGKGKREREKEKERTKLRERHRRA - 97
BAM8.2_Camelina_sativa_XP_010481609.1	Y	59 - GGGGKGKREREKEKERTKLRERHRRA - 84
BAM8.2_Camelina_sativa_XP_010494622.1	Y	76 - GGERGKGKREREKEKERTKLRERHRRA - 102
BAM8.2_S.indicum_XP_011082155.1	Y	66 - KSRKEREKEKERTKLRERHRRA - 87
BAM8_A.lyrata_XP_002865252.1	Y	76 - GGGGKGKREREKEKERTKLRERHRRA - 101
BAM8_A.thaliana_NP_199343.1	Y	79 - GGGGGKGKREREKEKERTKLRERHRRA - 105
BAM8_A.trichopoda_XP_011624011.1	Y	73 - KGRKEREKEKERTKLRERHRRA - 94
BAM8_B.rapa_XP_009128781.1	Y	72 - GGGKGKREREKEKERTKLRERHRRA - 96
BAM8_B.vulgaris_XP_010676684.1	Y	67 - GKGRREREKEKERTKLRERHRRA - 89
BAM8_C.annum_Capana01g004209	Y	66 - KSRKEREKEKERTKLRERHRRA - 87
BAM8_C.arietinum_XP_004512346.1	Y	46 - GKGKKEREKEKERTKLRERHRRA - 68
BAM8_C.cardunculus_KVH95140.1	Y	57 - SRNEREKEKERTKLRERHRR - 76
BAM8_C.clementia_XP_006432891.1	Y	65 - GKGKKEREKEKERTKLRERHRRA - 87
BAM8_C.lantus_Cla005462	Y	61 - GKAKREREKEKERTKLRERHRRA - 83
BAM8_C.melo_XP_008451866.1	Y	60 - GKAKREREKEKERTKLRERHRRA - 82
BAM8_C.rubella_XP_006279583.1	Y	89 - GGGGKGKREREKEKERTKLRERHRRA - 114
BAM8_C.sativus_XP_011653241.1	Y	61 - GKAKREREKEKERTKLRERHRRA - 83
BAM8_C.sinensis_AKQ62960.1	Y	48 - RRPRGFAASSSSGVAKGKKEREKEKERTKLRE
BAM8_C.sinensis_XP_006494107.1	Y	65 - GKGKKEREKEKERTKLRERHRRA - 87
BAM8_E.grandis_XP_010054915.1	Y	60 - PGKGKREREKEKERTKLRERHRRA - 83
BAM8_E.guttatus_XP_012837341.1	Y	68 - KSRKEREKEKERTKLRERHRRA - 89
BAM8_E.salsuginea_XP_006398198.1	Y	77 - GGGGGKGKREREKEKERTKLRERHRRA - 103
BAM8_F.vesca_XP_011465289.1	Y	54 - ISPSTKGRREREKEKERTKLRERLRR - 79
BAM8_G.max_XP_003516502.1	Y	53 - GGKGKKEREKEKERTKLRERHRRA - 76
BAM8_G.racemosa_QIKZ2029211	Y	70 - KGRKEREKEKERTKLRERHRRA - 91
BAM8_G.raimondii_XP_012439178.1	Y	46 - GKGKREREKEKERTKLRERHRRA - 68
BAM8_J.curcas_XP_012083880.1	Y	69 - GKGKREREKEKERTKLRERHRRA - 91
BAM8_M.acuminata_XP_009384530.1	Y	73 - AVAKGRKEREKEKERTKLRERHRRA - 97
BAM8_M.domestica_XP_008373437.1	Y	72 - KGKREREKEKERTKLRERLRR - 92
BAM8_M.notabilis_XP_010105162.1	Y	62 - SKGGKREREREKEKERTKLRERHRRA - 87
BAM8_M.trunculata_XP_003612541.1	Y	47 - GKGKKEREKEKERTKLRERHRRA - 69

BAM8_N.benthamiana_Nbv5tr6214148	Y	44 - AGATNKNRKEREKEKERTKLRERHRRA - 70
BAM8_N.nucifera_XP_010275178.1	Y	46 - GGKGKKEKRTKLRERHRRA - 64
BAM8_P.dactylifera_XP_008808792.1	Y	34 - AAAAGGSGKCRKEREKEKERTKLRERHRR - 62
BAM8_P.mume_XP_008238070.1	Y	91 - NKGKREREREKERTKLRERLRR - 112
BAM8_P.persica_XP_007210828.1	Y	69 - KGKREREREKERTKLRERLRR - 89
BAM8_P.trichocarpa_XP_002304400.1	Y	73 - GKGKREREKEKERTKLRERHRRA - 95
BAM8_P.vulgaris_XP_007158095.1	Y	55 - AKGKKEREKEKERTKLRERHRRA - 77
BAM8_R.communis_XP_002519919.1	Y	71 - RGKREREKEKERTKLRERHRRA - 92
BAM8_S.bicolor_XP_002451472.1	Y	16 - PPQRRPRGFASTAGGSPRRRGERERERERERTKL 56
BAM8_S.lycopersicum_XP_004244442.1	Y	60 - KSRKEREKEKERTKLRERHRRA - 81
BAM8_S.purpurea_SapurV1A.0961s0160	Y	76 - GKGKREREKEKERTKLRERHRRA - 98
BAM8_S.tuberosum_XP_006361593.1	Y	11 - KSRKEREKEKERTKLRERHRRA - 32
BAM8_T.cacao_XP_007040897.1	Y	60 - GKGKREREKEKERTKLRERHRRA - 82
BAM8_T.hassleriana_XP_010529274.1	Y	65 - GSGGGGAKGKREREKEKERTKLRERHRRA - 9
BAM8_V.vinifera_XP_002270680.1	Y	59 - GGGGGGKGKKEREKEKERTKLRERHRRA - 86
BAM8_Z.mays_XP_008679986.1	Y	19 - RRPRGFASAPAPAAGASPRRRGVQERERERERERKER - 62

Supplementary table 9: Predicted localisation of BAM8 orthologs

Name	Length	Score	cTP	CS-score	cTP-length
BAM9.1_A.lyrata_XP_002871828.1	534	0.445	-	0.548	15
BAM9.1_B.distachyon_XP_010229570.1	537	0.567	Y	6.588	64
BAM9.1_B.napus_XP_013730262.1	530	0.473	-	10.228	59
BAM9.1_B.rapa_XP_009131684.1	530	0.463	-	10.228	59
BAM9.1_Camelina_sativa_XP_010492888.1	533	0.484	-	9.325	55
BAM9.1_G.max_XP_003542915.1	536	0.473	-	2.186	14
BAM9.1_G.raimondii_XP_012454525.1	536	0.49	-	-0.54	64
BAM9.1_M.acuminata_XP_009399963.1	532	0.557	Y	6.836	67
BAM9.1_M.domestica_XP_008390741.1	529	0.471	-	7.893	62
BAM9.1_N.nucifera_XP_010241169.	541	0.464	-	8.481	73
BAM9.1_O.sativa_NP_001060573.1	523	0.468	-	6.389	16
BAM9.1_P.heterocycla_PH01003421G0090	488	0.44	-	7.157	35
BAM9.1_S.bicolor_XP_002463351.1	531	0.548	Y	6.25	51
BAM9.1_S.indicum_XP_011090854.1	539	0.542	Y	8.922	66
BAM9.1_S.italica_XP_004958614.1	524	0.537	Y	3.51	47
BAM9.1_S.polyrhiza_Spipo3G0042000	549	0.546	Y	10.142	63
BAM9.1_Z.mays_NP_001170007.1	531	0.56	Y	4.437	51
BAM9.2_A.lyrata_XP_002884575.1	453	0.441	-	12.423	62
BAM9.2_B.distachyon_XP_003561633.1	518	0.507	Y	4.715	20
BAM9.2_B.napus_XP_013683187.1	536	0.451	-	10.936	63
BAM9.2_B.rapa_XP_009120950.1	537	0.451	-	10.936	64
BAM9.2_Camelina_sativa_XP_010454120.1	533	0.485	-	10.228	62

BAM9.2_G.max_NP_001236364.1	536	0.472	-	2.186	14
BAM9.2_G.raimondii_XP_012482083.1	535	0.54	Y	7.604	59
BAM9.2_M.acuminata_XP_009391567.1	531	0.488	-	6.023	63
BAM9.2_M.domestica_XP_008340845.1	530	0.511	Y	8.901	63
BAM9.2_N.nucifera_XP_010245368.1	543	0.515	Y	2.044	52
BAM9.2_O.brachyantha_XP_006651377.1	299	0.495	-	4.264	11
BAM9.2_O.sativa_NP_001050116.2	524	0.504	Y	6.54	11
BAM9.2_P.heterocycla_PH01001710G0200	523	0.517	Y	3.19	6
BAM9.2_S.bicolor_XP_002467860.1	529	0.519	Y	7.081	51
BAM9.2_S.indicum_XP_011071485.1	539	0.542	Y	8.804	64
BAM9.2_S.italica_XP_004984382.1	521	0.488	-	8.519	49
BAM9.2_S.polyrhiza_Spipo22G0010700	556	0.504	Y	11.858	68
BAM9.2_Z.mays_NP_001151271.2	537	0.54	Y	8.352	55
BAM9.3_Camelina_sativa_XP_010420647.1	533	0.474	-	9.325	55
BAM9_A.corulea_Aquca_003_00854	532	0.484	-	2.118	60
BAM9_A.thaliana_NP_197368.1	536	0.441	-	9.325	55
BAM9_A.trichopoda_XP_006855410.1	524	0.431	-	2.733	9
BAM9_B.vulgaris_XP_010666684.1	539	0.476	-	6.595	66
BAM9_C.annum_Capana01g003107	534	0.503	Y	1.067	60
BAM9_C.arietinum_XP_004486065.1	536	0.514	Y	1.398	33
BAM9_C.canephora_CDO98919.1	540	0.478	-	8.253	66
BAM9_C.cardunculus_KVH91414.1	819	0.467	-	3.725	55
BAM9_C.clementia_XP_006419671.1	543	0.486	-	8.526	26
BAM9_C.lantus_Cla009332	532	0.513	Y	5.572	55
BAM9_C.melo_XP_008458491.1	533	0.521	Y	3.782	55
BAM9_C.papaya_evm.model.supercontig_16.112	546	0.471	-	10.076	72
BAM9_C.rubella_XP_006287474.1	532	0.479	-	7.803	63
BAM9_C.sativus_XP_004153140.1	532	0.51	Y	5.572	55
BAM9_C.sinensis_XP_006489160.1	543	0.483	-	8.526	26
BAM9_D.carota_KZM87479.1	532	0.483	-	5.188	57
BAM9_E.grandis_XP_010024561.1	532	0.511	Y	2.64	55
BAM9_E.guineesis_XP_010938702.1	530	0.485	-	9.64	60
BAM9_E.guttatus_XP_012827989.1	374	0.55	Y	5.426	67
BAM9_E.salsuginea_XP_006400419.1	533	0.449	-	9.325	55
BAM9_F.vesca_XP_004296793.1	530	0.479	-	2.908	54
BAM9_G.racemosa_QIKZ2016136	526	0.573	Y	6.215	53
BAM9_H.vulgare_BAK03717.1	526	0.516	Y	0.841	46
BAM9_I.vomitoria_ASMV2109307	540	0.505	Y	9.347	65
BAM9_J.curcas_XP_012069407.1	532	0.499	-	6.627	69
BAM9_M.esculenta_cassava4.1_005239m	535	0.506	Y	0.987	72
BAM9_M.notabilis_XP_010105020.1	535	0.49	-	8.398	64
BAM9_M.trunculata_XP_003594004.1	535	0.448	-	6.297	64
BAM9_N.benthamiana_Nbv5tr6214300	540	0.482	-	3.038	64
BAM9_P.dactylifera_XP_008796202.1	524	0.471	-	7.987	60
BAM9_P.mume_XP_008223100.1	530	0.485	-	8.901	62
BAM9_P.persica_XP_007222488.1	529	0.489	-	8.901	62

BAM9_P.trichocarpa_XP_002312750.2	535	0.519	Y	3.265	60
BAM9_P.vulgaris_XP_007147864.1	532	0.493	-	5.875	64
BAM9_R.communis_XP_002516865.1	545	0.516	Y	6.222	81
BAM9_S.lycopersicum_NP_001234052.1	535	0.56	Y	3.038	65
BAM9_S.purpurea_SapurV1A.0022s0350	535	0.566	Y	7.113	60
BAM9_S.tuberosum_XP_006342739.1	535	0.558	Y	3.038	65
BAM9_T.cacao_XP_007035476.1	537	0.491	-	2.061	71
BAM9_T.hassleriana_XP_010558493.1	536	0.46	-	12.7	62
BAM9_V.vinifera_XP_002276777.1	541	0.49	-	6.827	65

Supplementary table 10: Predicted localisation of BAM9 orthologs

Name	Length	Score	cTP	CS-score	cTP-length
BAM10.1_G.max_XP_003532447.1	553	0.58	Y	2.400	40
BAM10.1_M.domestica_XP_008390323.1	559	0.57	Y	1.909	59
BAM10.1_Z.mays_NP_001130896.1	539	0.59	Y	6.177	47
BAM10.2_G.max_XP_003525331.1	557	0.57	Y	0.527	49
BAM10.2_M.domestica_XP_008337562.1	559	0.57	Y	6.923	59
BAM10.2_Z.mays_NP_001132696.1	537	0.59	Y	1.833	45
BAM10_A.duranensis_Aradu.FYP9T.1	544	0.58	Y	2.331	59
BAM10_A.trichopoda_XP_006844925.1	559	0.58	Y	5.275	54
BAM10_B.distachyon_XP_003566188.1	556	0.58	Y	3.029	45
BAM10_C.arietinum_XP_004503587.1	554	0.57	Y	0.970	40
BAM10_C.clementia_XP_006439286.1	541	0.56	Y	3.872	99
BAM10_C.lantus_Cla002226	548	0.58	Y	0.448	50
BAM10_C.melo_XP_008460412.1	546	0.58	Y	0.448	50
BAM10_C.sativus_XP_004144400.1	546	0.58	Y	0.564	36
BAM10_Camellia.sinensis_AKQ62958.1	549	0.56	Y	5.177	27
BAM10_Citrus.sinensis_XP_006476339.1	536	0.56	Y	3.872	99
BAM10_E.grandis_XP_010055984.1	551	0.56	Y	3.066	31
BAM10_E.guineesis_XP_010915994.1	550	0.57	Y	1.944	66
BAM10_E.guttatus_XP_012842111.1	518	0.54	Y	4.132	49
BAM10_F.vesca_XP_004301815.1	542	0.58	Y	3.347	46
BAM10_H.vulgare_BAJ96156.1	547	0.57	Y	7.440	32
BAM10_J.curcas_XP_012086671.1	553	0.59	Y	3.709	50
BAM10_M.acuminata_XP_009403535.1	542	0.56	Y	1.529	44
BAM10_M.acuminata_XP_009409087.1	561	0.56	Y	1.997	74
BAM10_M.notabilis_XP_010107262.1	560	0.57	Y	1.230	19
BAM10_M.trunculata_XP_013447245.1	545	0.58	Y	1.909	47
BAM10_N.benthamiana_Nbv5tr6219815	548	0.57	Y	2.301	49
BAM10_O.sativa_NP_001172248.1	587	0.58	Y	2.532	66
BAM10_P.dactylifera_XP_008783150.1	550	0.56	Y	1.697	55
BAM10_P.mume_XP_008239169.1	567	0.58	Y	3.766	57
BAM10_P.persica_XP_007209090.1	567	0.58	Y	3.766	57
BAM10_P.trichocarpa_XP_002297961.1	555	0.58	Y	1.286	43

BAM10_P.vulgaris_XP_007160198.1	549	0.57	Y	-0.451	42
BAM10_S.indicum_XP_011070357.1	538	0.55	Y	4.132	51
BAM10_S.italica_XP_004967358.1	544	0.59	Y	2.132	42
BAM10_S.lycopersicum_XP_004245482.1	539	0.57	Y	4.131	47
BAM10_S.polyrhiza_Spipo8G0049600	568	0.58	Y	5.189	47
BAM10_S.tuberosum_XP_006343811.1	541	0.57	Y	4.976	47
BAM10_T.cacao_XP_007040595.1	627	0.57	Y	1.952	62
BAM10_V.vinifera_XP_010659745.1	542	0.56	Y	0.893	48

Supplementary table 11: Predicted localisation of BAM10 orthologs

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7 – Blue Light Induces a Distinct Starch Degradation Pathway in Guard Cells for Stomatal Opening

Daniel Horrer, Sabrina Flütsch, Diana Pazmino, Jack SA Matthews, Matthias Thalmann, Arianna Nigro, Nathalie Leonhardt, Tracy Lawson, Diana Santelia

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Synopsis: While the focus of my PhD was the involvement of AMY3 and BAM1 in stress responses, both enzymes also participate in starch degradation in guard cells. In this report, it was shown that starch is rapidly degraded in guard cells upon illumination. This starch degradation is mediated by AMY3 and BAM1, and the double mutant is not only deficient in starch degradation but also light-induced stomatal opening.

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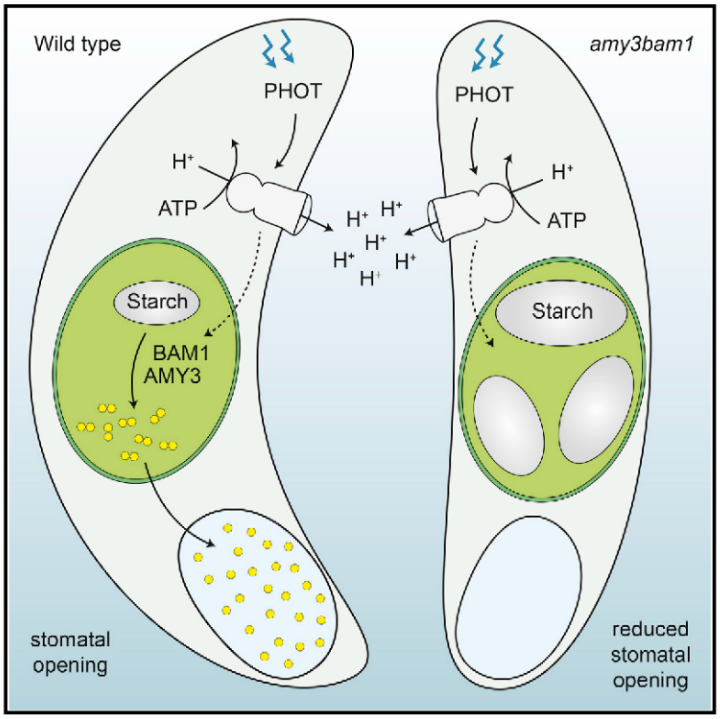
Report

Current Biology

Blue Light Induces a Distinct Starch Degradation Pathway in Guard Cells for Stomatal Opening

Graphical Abstract



Highlights

- Starch in guard cells is degraded within 30 min of light to promote stomatal opening
- A distinct set of hydrolytic enzymes mediates starch degradation in guard cells
- Phototropin signaling and proton pumping control starch degradation in guard cells
- Guard cell starch drives plant growth through the control of stomatal aperture

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In Brief

Horrer et al. report quantitative analysis of starch turnover in guard cells of intact *Arabidopsis* leaves and show it to be an ideal system to study guard cell carbohydrate metabolism. Molecular genetic analyses show that guard cells integrate blue-light-induced proton pumping with starch degradation to control stomatal opening and plant growth.

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Current Biology
Report

Blue Light Induces a Distinct Starch Degradation Pathway in Guard Cells for Stomatal Opening

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SUMMARY

Stomatal pores form a crucial interface between the leaf mesophyll and the atmosphere, controlling water and carbon balance in plants [1]. Major advances have been made in understanding the regulatory networks and ion fluxes in the guard cells surrounding the stomatal pore [2]. However, our knowledge on the role of carbon metabolism in these cells is still fragmentary [3–5]. In particular, the contribution of starch in stomatal opening remains elusive [6]. Here, we used *Arabidopsis thaliana* as a model plant to provide the first quantitative analysis of starch turnover in guard cells of intact leaves during the diurnal cycle. Starch is present in guard cells at the end of night, unlike in the rest of the leaf, but is rapidly degraded within 30 min of light. This process is critical for the rapidity of stomatal opening and biomass production. We exploited *Arabidopsis* molecular genetics to define the mechanism and regulation of guard cell starch metabolism, showing it to be mediated by a previously uncharacterized pathway. This involves the synergistic action of β-amylase 1 (BAM1) and α-amylase 3 (AMY3)—enzymes that are normally not required for nighttime starch degradation in other leaf tissues. This pathway is under the control of the phototropin-dependent blue-light signaling cascade and correlated with the activity of the plasma membrane H^+-ATPase. Our results show that guard cell starch degradation has an important role in plant growth by driving stomatal responses to light.

RESULTS

Starch Dynamics in Guard Cells of Intact *Arabidopsis* Leaves

Starch is a complex glucose polymer found in plastids of higher plants [7]. In guard cell chloroplasts, starch has long been implicated in light-induced stomatal opening. Outlaw and Manchester showed 35 years ago that guard cell starch concentration was higher in leaflets of *Vicia faba* with closed stomata than with open stomata [8]. Schnabl showed a correlation between changes in volume of *Vicia faba* guard cell protoplasts and proposed the interconversion of malate and starch [9]. Talbott and Zeiger observed an increase in sucrose levels in *Vicia faba* epidermal peels throughout the light-stimulated stomatal opening concomitantly with the accumulation of the starch hydrolytic products maltose and maltotriose [10]. These observations led to the hypothesis that guard cell starch is mobilized in the light to generate malate and/or sucrose to sustain stomatal opening. After these early publications, the literature on the subject has been fragmentary and mostly correlative in nature, due to the lack of suitable methods and genetic material. Changes in guard cell starch concentrations were observed in other species (*Ocimum basilicum* and *Commelina communis*), but no details about the temporal dynamics or the metabolic pathway were reported [4, 11]. In *Arabidopsis thaliana*—the most-well-studied model plant—there is no clear evidence of starch breakdown in guard cells in the light. Lasceve et al. showed electron micrographs of starch granules in *Arabidopsis* guard cells at the end of the night [12], suggesting that starch metabolism follows an opposite rhythm compared to mesophyll cells, in which starch is degraded at night [13]. In contrast, Stadler and coworkers reported that starch was nearly absent in *Arabidopsis* guard cells at the end of the night [14]. Recently, Daloso et al. suggested that in tobacco guard cells, changes in sugars—not starch—drive light-induced stomatal opening [15]. Due to these contradictory reports, guard cell starch metabolism is currently thought to differ between species [6], and its genetic and molecular basis remains mostly unknown.

Until now, starch content in guard cells has been generally estimated on a relative value scale by comparing the intensity of iodine-stained guard cell chloroplasts [11, 16–18]. A few studies have determined guard cell starch content quantitatively using the "oil-well technique" on freeze-dried leaflets [8, 19] or spectrophotometrically using guard-cell-enriched epidermal fragments [15]. In such cases, however, no details of temporal dynamics over the diurnal cycle were provided. Here, we developed a new method to quantify starch in guard cells of intact leaves. Epidermal peels from mature leaves were immediately fixed and treated with periodic acid, and the glucans were covalently linked to the fluorophore propidium iodide. Confocal microscopy then enabled high-resolution analytical imaging to quantify the starch in guard cells over

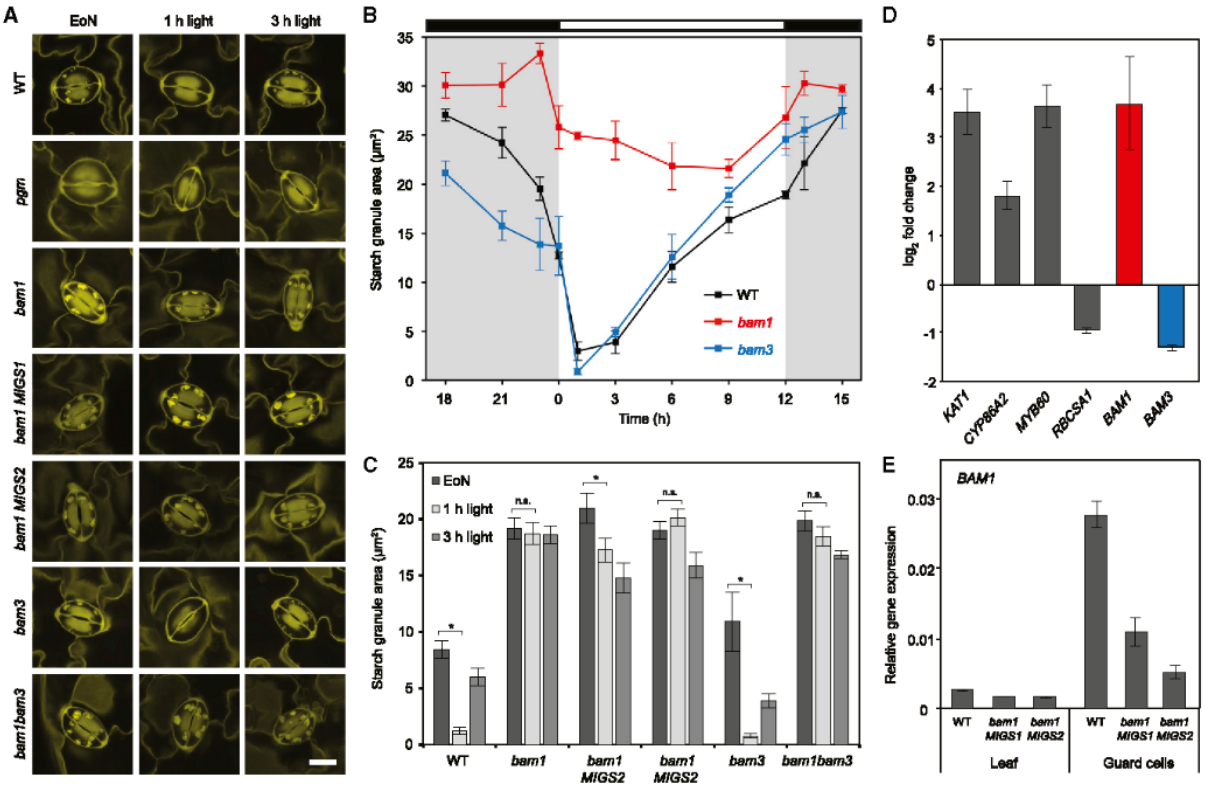


Figure 1. Starch Turnover in *Arabidopsis* Guard Cells and Isoform Specialization within the β-Amylase Family

(A) Visualization of starch granules within *Arabidopsis* guard cells of intact leaves at the end of the night (EoN) and after 1 hr and 3 hr of illumination. Scale bar, 10 µm.

(B) Starch accumulation in guard cells of intact leaves over the diurnal cycle (n = 110; ±SEM).

(C) Starch accumulation in guard cells of intact leaves at the EoN and after 1 hr and 3 hr of illumination (n = 100; ±SEM). An unpaired Student's t test determined statistical significance between EoN and 1 hr light (*p < 0.05; n.s., not significant).

(D) Gene transcript abundance in guard-cell-enriched epidermal peel fragments compared to leaves. Values are normalized against gene expression in the leaves (set as 0; n = 3; ±SEM).

(E) Relative expression levels of *BAM1* in leaves and guard-cell-enriched epidermal peel fragments of the WT and two independent *BAM1* MIGS silencing lines (n = 3; ±SEM). *BAM1* transcripts in the guard cells of the two silencing lines are reduced by 60% and 80%, respectively, but are unaffected in the leaves.

See also Figure S1.

the diurnal cycle. This analysis was performed in *Arabidopsis* because this plant is widely used as a model system for guard cell physiology, because of the contradictory literature regarding its guard cell starch metabolism, and to exploit the wealth of molecular genetic resources. As for other species [4, 8, 11], starch was present in *Arabidopsis* wild-type (WT) guard cells at the end of the night (Figures 1A–1C). In response to light, starch was rapidly degraded within 1 hr (Figures 1A–1C). As expected, guard cells of the *Arabidopsis* starch-deficient mutant *pgm* (*PHOSPHOGLUCOMUTASE 1*) were devoid of starch (Figure 1A) [12]. Interestingly, in WT plants, starch started to decline during the latter part of the night, and by dawn about half was gone. However, nighttime degradation occurred slowly compared to the light-induced degradation (Figure 1B). Starch synthesis started 1 hr into the light, was maintained for the rest of the photoperiod, and continued into the night (Figure 1B). These results show that *Arabidopsis* guard cell starch metabolism differs markedly from the rest of the leaf, where starch is synthesized during the day and degraded at night in a near-linear manner [20].

BAM1 and BAM3 Have a Cell-Type-Specific Function

Next, we investigated the enzymes responsible for starch degradation in guard cells. β-amylase (BAM), which releases maltose from glucans, is the main starch-degrading enzyme [7, 13]. Mutant studies suggest that BAM3 is the major isoform in leaf mesophyll starch metabolism, whereas BAM1 has limited or no involvement [21]. Recently, it was shown that *bam1* mutants have more starch in illuminated guard cells and reduced stomatal opening compared to the WT [18], resulting in drought tolerance [17]. Consistently, we found that *BAM1* was preferentially and highly expressed in guard-cell-enriched epidermal peels, similarly to established markers for guard-cell-specific expression; the inward-rectifying K^+ *CHANNEL IN ARABIDOPSIS*

THALIANA 1 (KAT1), CYTOCHROME P450 86A2 (CYP86A2), and MYB TRANSCRIPTION FACTOR 60 (MYB60) (Figures 1D and 1E) [22, 23]. Compared with the WT, bam1 mutants displayed a guard-cell-specific starch excess (sex) phenotype throughout the diurnal cycle (Figures 1A–1C, and S1). To provide direct genetic evidence that the mutation in BAM1 was responsible for the guard cell sex phenotype, we repressed BAM1 in guard cells of the WT using microRNA-induced gene silencing (MIGS) [24], driven by the CYP86A2 promoter [23]. BAM1 silencing lines showed reduced guard cell BAM1 transcript levels and increased starch content, mimicking the bam1 phenotype (Figures 1A, 1C, and 1E). These results show that, under standard growth conditions, BAM1 functions specifically in guard cell starch metabolism. In contrast, BAM3 transcripts were considerably less abundant in guard cells (Figure 1D), and guard cell starch levels in bam3 were similar to those in the WT (Figures 1A–1C). This suggests that BAM1 and BAM3 acquired different cell-type-specific functions. Interestingly, bam1bam3 double mutants had similar guard cell starch content to the bam1 single mutants (Figures 1A and 1C). This contrasts to the situation in mesophyll cells, where bam1bam3 has a more severe sex phenotype than bam3 single mutants (Figure S1) [21]. These data reveal that sub-functionalization among the chloroplastic BAMs enables the plant to adjust starch turnover to the needs of the individual cell.

A Distinct Pathway of Starch Degradation Operates in Guard Cells during Light-Induced Stomatal Opening

Given the complex nature of starch polymers, β-amylases need to work with other glucan hydrolases to accomplish complete degradation [13, 25, 26]. We hypothesized that other enzymes that have no clear function in mesophyll starch metabolism may have a role in guard cells. As for BAM1, loss of α-AMYLASE 3 (AMY3) and the debranching enzyme LIMIT DEXTRINASE (LDA) do not affect starch metabolism in the leaves (Figure S1) [27, 28]. Interestingly, AMY3 was strongly upregulated in guard cells (Figure 2A), and the amy3 mutant showed a slight but significant and reproducible guard cell sex phenotype (Figures 2B and 2C). Loss of both AMY3 and BAM1 proteins resulted in a much higher starch content than in the bam1 mutant (Figures 2B and 2C), without affecting starch metabolism in the leaves (Figure S1). This striking amy3bam1 phenotype indicates a role for AMY3 in guard cell starch metabolism. Unlike AMY3 and BAM1, LDA was not upregulated in guard cells (Figure 2A). Mutation of LDA had a marginal, albeit significant and reproducible, impact on guard cell starch metabolism (Figures 2B and 2C). However, transcript abundance of ISOAMYLASE 3 (ISA3)—the major debranching enzyme in nighttime leaf starch degradation—was 7-fold higher in guard-cell-enriched epidermal peels relative to intact leaves (Figure 2A). Guard cells of isa3 contained elevated starch compared with the WT, even though appreciable starch degradation still occurred upon illumination (Figures 2B and 2C). The isa3 phenotype was exacerbated by the additional mutation of LDA (isa3lda; Figures 2B and 2C), reproducing in guard cells the situation previously described in leaves (Figure S1) [28]. Collectively, these results indicate that BAM1, AMY3, LDA, and ISA3 work together toward efficient guard cell starch mobilization during stomatal opening.

Mutations in the Stomatal Starch Degradation Pathway Impact Plant Growth

To investigate the importance of starch degradation in guard cells, we examined the consequences of the absence of BAM1 and AMY3 on stomatal function and whole-plant physiology. Intact leaves of bam1 mutants showed reduced stomatal aperture and slower increases in stomatal conductance (g_s) in response to light compared with the WT (Figures S2A and S2B). This correlates with the lack of light-induced starch degradation in bam1 guard cells (Figures 1A–1C). However, CO_2 assimilation (A) was generally not affected in bam1 plants, with only subtle differences during the first 1 hr of light (Figure S2C). In contrast to bam1, bam3 mutants had normal rate of stomatal opening in response to light. Transpiration and carbon assimilation rates were also normal (Figures S2D–S2F) indicating that BAM3 is not required for proper stomatal function.

The additional loss of AMY3 exaggerated the suppression of plant stomatal responses. First, amy3bam1 plants failed to open stomata efficiently in response to light (Figure 3A). Consequently, amy3bam1 had lower g_s (Figure 3B), decreased leaf intercellular CO_2 concentration (C_i; Figure 3C), and slightly lower CO_2 assimilation rates (Figure S2G) immediately after illumination compared with the WT. This suppression of stomatal function in the amy3bam1 double mutant had consequences for plant growth, especially under higher light intensities. 4-week-old amy3bam1 plants grown under 120 μmol m^{-2} s^{-1} of light were almost 30% smaller than the WT, whereas there were no noticeable differences when plants were kept under low light intensities (Figure 3D and Figure S2H). These results suggest that diffusive (stomatal) limitations in amy3bam1 plants may limit CO_2 availability for mesophyll photosynthesis upon illumination, at least under higher light intensities. Thus, we conclude that guard cell starch degradation is of major importance in plant adaptation to light through the control of stomatal aperture.

Guard Cell Starch Degradation Is Specifically Activated by Blue Light through the PHOT1/PHOT2-Dependent Signaling Pathway

Starch degradation in guard cell chloroplasts is believed to occur mainly under blue light [5, 10], as the Arabidopsis pgm mutant shows a reduced rate of stomatal opening, which seems largely attributable to the inhibition of blue-light-induced mechanisms [12]. Consequently, we investigated whether blue light is the actual signal for guard cell starch degradation. We found that starch was efficiently degraded and stomata opened in Arabidopsis WT plants illuminated with 75 μmol m^{-2} s^{-1} of blue light, a stimulus insufficient to activate photosynthesis at significant levels (Figures 4A, S3A, and S3B). In contrast, illumination with 300 μmol m^{-2} s^{-1} of red light promoted efficient starch synthesis in guard cells and induced stomatal opening independently of starch (Figures 4A and S3A). Thus, distinct light qualities control synthesis and degradation of starch in guard cells, and stomatal opening triggered by red light seems to be largely independent of the breakdown of starch present in guard cell chloroplasts.

In guard cells, blue-light perception by PHOTOTROPIN 1 and 2 (PHOT1/PHOT2) [29] initiates a signal transduction cascade, involving the BLUE LIGHT SIGNALING 1 (BLUS1) protein kinase [30] and PROTEIN PHOSPHATASE 1 (PP1) [31, 32]. The signal ultimately activates the plasma membrane H^+-ATPase, leading

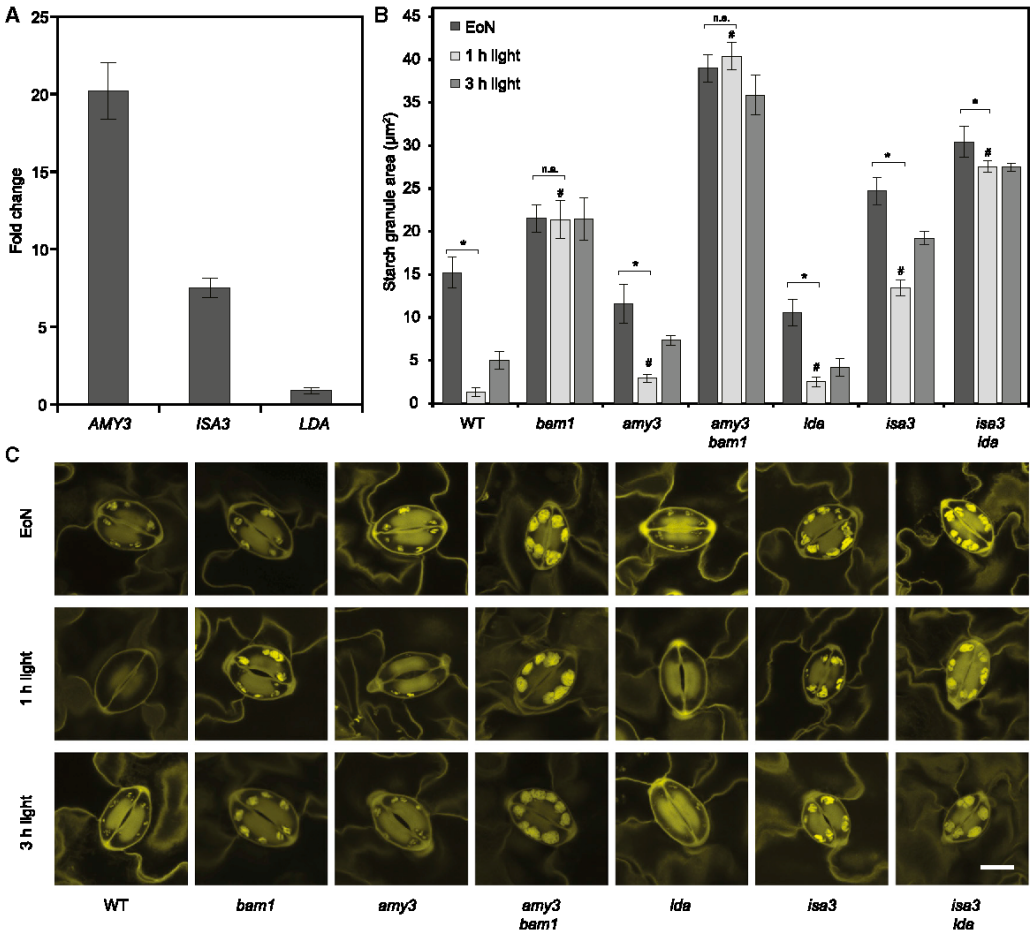


Figure 2. BAM1, AMY3, ISA3, and LDA Form a Distinct Pathway of Starch Degradation in *Arabidopsis* Guard Cells

(A) Transcript abundance of *AMY3*, *ISA3*, and *LDA* in guard-cell-enriched epidermal peel fragments compared to leaves. Values are normalized against gene expression in the leaves (set as 1; n = 3; ±SEM).

(B) Quantification of starch content in guard cells of intact leaves at the EoN and after 1 hr and 3 hr of illumination (n = 110; ±SEM). Statistical significances were determined by unpaired Student's t tests: #$p < 0.05$ mutants versus WT at the indicated time point; *$p < 0.05$ for the indicated comparisons; n.s., not significant for the indicated comparison.

(C) Representative confocal images of propidium-iodide-stained guard cells of intact leaves. Scale bar, 10 µm.

See also Figure S1.

to increased inside-negative electric potential, influx of potassium and water, and opening of the stomatal pore [2, 33–35]. To determine the molecular basis of blue-light-induced guard cell starch degradation, we investigated starch turnover in the *Arabidopsis* blue-light-signaling mutants *phot1phot2* and *blus1*. In both mutants, guard cells of intact leaves still contained high levels of starch after 1 hr of light, whereas the WT and the *blus1::GFP-BLUS1* complementing line efficiently degraded it (Figures 4B, 4C, S3C, and S3D). In fact, the WT consumed almost all the starch within 30 min of light (Figure 4B), showing that the response of guard cell starch degradation to light is extremely rapid. In contrast to guard cells, synthesis and degradation of starch in *phot1phot2* and *blus1* mesophyll cells occurred normally (Figures S3E and S3F), even though the overall starch levels in *phot1phot2* leaves were reduced compared to the WT (Figure S3E). This is most likely a consequence of the impaired CO_2 assimilation rate in this mutant [36]. Tautomycin is a potent inhibitor of PP1, which mediates blue-light signaling between phototropin and the plasma membrane H^+-ATPase [32]. Consistent with a role for blue-light signaling in guard cell starch degradation, we found that tautomycin completely blocked starch degradation and stomatal opening (Figures 4D and S3G). Altogether, these results indicate that the effect of blue light on starch degradation is phototropin dependent and specific to guard cells.

Proton Extrusion by H^+-ATPase Is Required for Starch Degradation during Stomatal Opening

Given that the plasma membrane H^+-ATPase is the ultimate target of the blue-light signaling cascade [33], we investigated

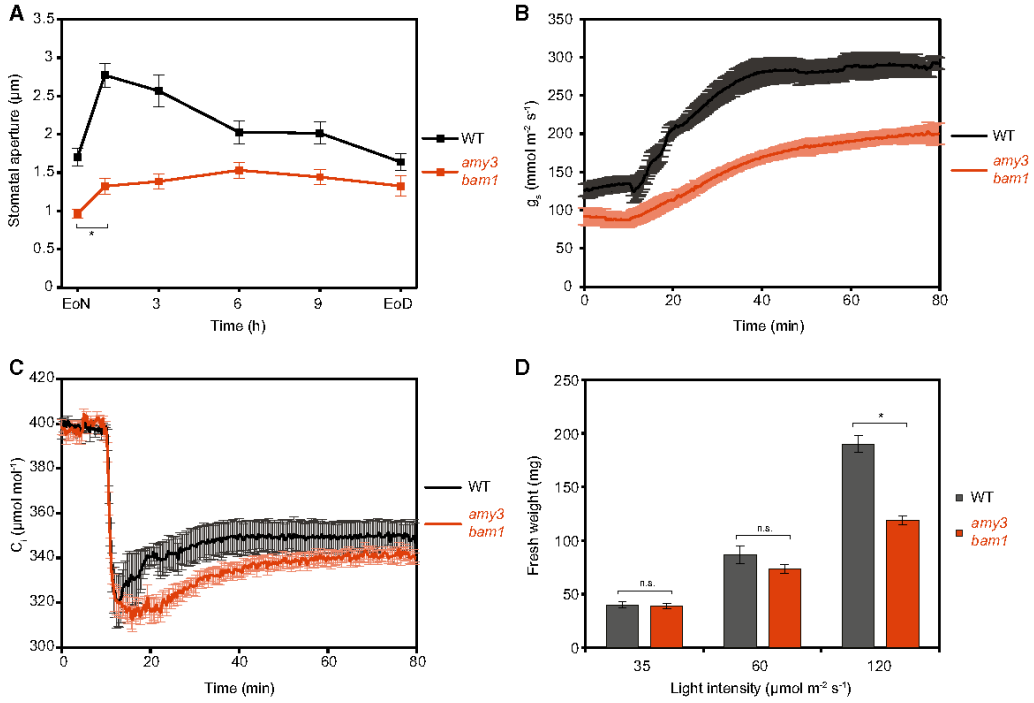


Figure 3. Stomatal Function and Biomass Production in Response to Light Are Impaired in *amy3bam1* Mutants

(A) Stomatal aperture in WT and *amy3bam1* intact leaves during the light phase, determined by light microscopy and digital image processing (n = 100; ±SEM). Statistical significances were determined by unpaired Student's t tests: *p < 0.01 for the indicated comparison.

(B and C) Single leaf measurement of WT and *amy3bam1* stomatal conductance (g_s; B) and internal CO_2 concentration (C) in 150 µmol m^{-2} s^{-1} light intensity (n ≥ 5; ±SEM).

(D) Fresh weight of WT and *amy3bam1* rosettes of 4-week-old plants grown at different light intensities (n = 5; ±SEM). Statistical significances were determined by unpaired Student's t tests: *p < 0.01 for the indicated comparison; n.s., not significant for the indicated comparison.

See also Figure S2.

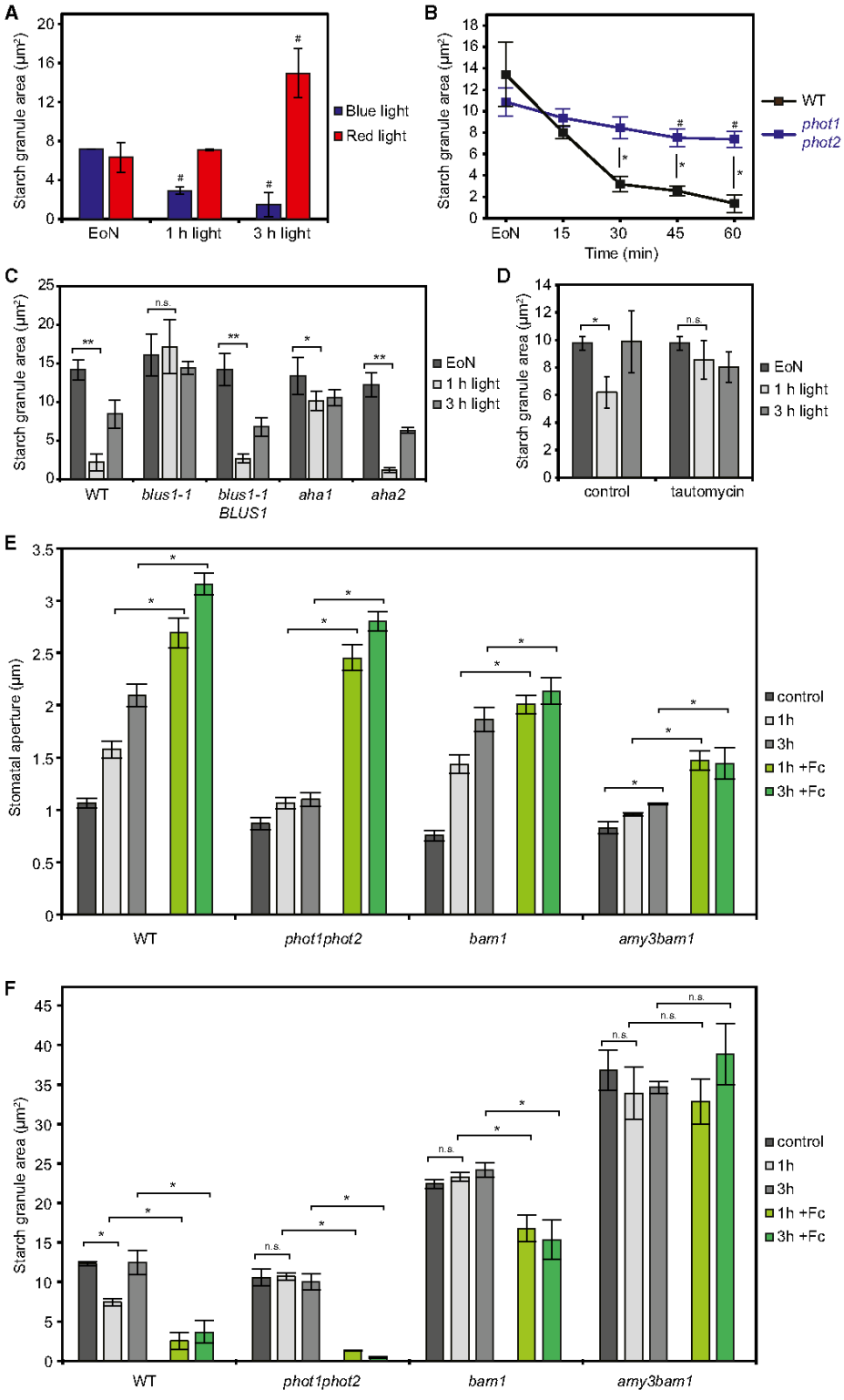
whether proton-pump activity was required for guard cell starch breakdown. Mutation of plasma membrane H$^+$-ATPase1 (*aha1*) resulted in a severe guard cell *sex* phenotype without affecting leaf starch metabolism (Figures 4C and S3F). In contrast, mutants of H$^+$-ATPase2 (*aha2*) displayed a WT phenotype (Figure 4C), suggesting a major role for AHA1 in guard cells, although both isoforms are expressed [37].

The fungal toxin fusicoccin (Fc) is a well-known chemical activator of the proton pump [38]. As expected, stomata in WT isolated epidermal peels floating in a buffer containing 50 mM KCl opened to a greater extent in the presence of 10 µM Fc (Figure 4E) [29]. Starch in guard cell chloroplasts in isolated epidermal peels floating in control buffer showed a similar pattern of light-induced starch degradation and re-synthesis to that of guard cells of intact leaves (Figure 4F versus Figure 1C). However, upon treatment with Fc, almost all guard cell starch disappeared after 1 hr (Figure 4F), suggesting that enhancement of the H$^+$-ATPase activity correlates with enhanced starch degradation. A similar pattern of stomatal opening and guard cell starch accumulation was found in *phot1phot2* after Fc treatment (Figures 4E and 4F). On the basis of these results, we conclude that proton extrusion across the plasma membrane during stomatal opening is required for guard cell starch breakdown and that the activity of the H$^+$-ATPase is positively correlated with starch degradation.

Stomata in *bam1* isolated epidermal peels floating in the buffer containing 50 mM KCl opened similarly to that of the WT, most likely due to the presence of high concentration of Cl$^-$ in the medium (Figure 4E). The presence of Cl$^-$, however, had only little effect on *amy3bam1* stomatal opening (Figure 4E). Exogenous application of Fc only partially rescued stomatal opening and guard cell starch degradation in *bam1* and had little or no effect on *amy3bam1* (Figures 4E and 4F). These results indicate that H$^+$-ATPase functions upstream of starch degradation and that this process is ultimately required for blue-light-induced stomatal opening.

DISCUSSION

It has been 100 years since the first observation that starch metabolism in guard cell chloroplasts follows a different rhythm compared to other photosynthetically active leaf tissues [39]. However, despite the increasing interest in guard cell function and regulation, the role of starch metabolism in guard cells and its influence on stomatal function remains only poorly understood, especially in *Arabidopsis*. Our new protocol to quantify



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starch in single cells showed that starch is present in *Arabidopsis* guard cells at the end of the night, unlike the rest of the leaf. Upon illumination, the starch is very rapidly degraded and most is gone within 30 min (Figures 1B and 4B). Thus, we conclude that starch metabolism in *Arabidopsis* guard cells is similar to that of other species [4, 8, 11], overcoming the contradictions in the literature [12, 14].

By conducting *Arabidopsis* mutant analyses, we discovered that light-induced starch degradation in guard cells is surprisingly mediated by a previously uncharacterized pathway, which involves the activity of BAM1 and AMY3, which are normally not required for nighttime starch degradation in the rest of the leaf (Figures 1, 2, and S1). We propose that a key mechanism underpinning such plasticity in starch mobilization is enzyme isoform specialization. In support of this, we showed that two β-amylase isoforms have acquired tissue-specific function. BAM3 has low expression in guard cells (Figure 1D) but represents the major isoform in the nighttime leaf starch degradation (Figure S1) [21]. BAM1 is highly expressed in guard cells and is required for light-induced starch degradation in these cells (Figures 1 and S1) [18]. Interestingly, BAM1 is redox regulated, whereas BAM3 is not, and the cysteines involved in the disulfide formation in BAM1 are not conserved in BAM3 [40, 41]. It is therefore plausible to imagine that redox control of BAM1 may activate starch degradation in the light when the cellular environment becomes more reducing due to the activity of the electron transport chain [42]. We recently showed that, similarly to BAM1, AMY3 is also a redox regulated enzyme [26], strengthening the concept that BAM1 and AMY3 are co-regulated and work synergistically. Blocking starch degradation in guard cells by simultaneous loss of BAM1 and AMY3 proteins has severe consequences for stomatal function and a perceptible impact on plant growth, especially under high light intensities (Figure 3). This knowledge reveals the importance of starch in guard cells in driving plant growth through the control of stomatal movements.

Lastly, we provide compelling evidence that this newly discovered starch degradation pathway is under the direct control of blue light through the PHOT1/PHOT2-dependent signaling cascade. Mutation in the blue-light-signaling components PHOT1/PHOT2 or BLUS1 impacted starch degradation in guard cells (Figures 4B and 4C), but not in mesophyll cells (Figures S3E and S3F), demonstrating that the influence of blue light on starch metabolism is specific to guard cells. Furthermore, we provide evidence of a direct link between proton pumping and starch degradation in guard cells. The inability of the *aha1* mutants to mobilize starch in guard cells efficiently upon illumination (Figure 4C), along with the low sensitivity of *amy3bam1* guard cells to Fc treatment (Figures 4E and 4F), show that proton pumping is required for starch degradation during stomatal opening. Interestingly, a previous study showed a similar correlation between proton pumping and the activity of the guard cell isoform of *PHOSPHOENOLPYRUVATE CARBOXYLASE* (*PEPC*), which is required for the synthesis of malate [43]. This observation points toward a coordinated activation of the blue-light-dependent mechanisms required for stomatal opening, whereby maltose originated from starch degradation is converted to malate in the cytosol through glycolysis and the action of PEPC, with an estimated net production of four molecules of malate and one molecule of ATP per molecule of maltose (Data S1). Considering the malate concentration of open guard cells to be around 100–200 mM [44], we propose that approximately half of the starch broken down during early stomatal opening is sufficient for rapid malate synthesis (Data S1). The fate of the remainder carbon skeletons is not clear, but they are presumably used for sucrose or other hexose sugars production as additional osmolytes or for cellular respiration to produce the ATP required for H^+ pumping.

The signal ultimately linking plasma membrane H^+-ATPase activity to starch degradation in the chloroplast is unknown. The alkalinization of the cytosol resulting from proton pumping upon illumination stimulates accumulation of H^+ through malate synthesis [45], a process requiring carbon skeletons deriving from starch degradation [9, 46]. Depletion of protons in the cytosol may therefore represent a signal for guard cell starch degradation in response to light, although it is difficult to imagine how changes in cytosolic pH are transferred to the chloroplast. Alternatively, changes in metabolic fluxes upon activation of stomatal opening may trigger starch degradation indirectly, through metabolic signals such as the increase in ADP:ATP ratio or the depletion of the glycolysis intermediate PEP. Lastly, both BAM1 and AMY3 are preferentially active at a slightly alkaline pH [26, 41, 47]. Thus, the reducing environment and the alkaline pH of the stroma generated by the photosynthetic electron transport chain in the light [42, 48] would favor BAM1 and AMY3 activity, promoting starch degradation.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one dataset and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.12.036.

Figure 4. Blue-Light Signaling and Proton Pumping Activate Guard Cell Starch Degradation during Stomatal Opening

(A) Starch content in guard cells of WT plants illuminated with blue light (75 μmol m^{-2} s^{-1}) or red light (300 μmol m^{-2} s^{-1}) after the end of the dark period (n = 110; ±SEM). An unpaired Student's t test determined statistical significance between EoN and 1 hr and 3 hr light (#$p < 0.01$).

(B) Guard cell starch content of WT and *phot1phot2* intact leaves during the first hour of the light period (n = 110; ±SEM). Statistical significances were determined by unpaired Student's t tests: *$p < 0.001$ for the indicated comparison; #$p < 0.001$ between EoN and 45 min and 60 min of light for the *phot1phot2* mutant.

(C) Starch accumulation in guard cells of intact leaves at the EoN and after 1 hr and 3 hr of illumination (n = 110; ±SEM). An unpaired Student's t test determined statistical significance between the indicated comparisons (*$p < 0.01$; **$p < 0.001$; n.s., not significant).

(D) Guard cell starch content in WT epidermal peels treated for 1 hr and 3 hr with or without 2.5 μM tautomycin in opening buffer containing 50 mM KCl. 1 hr dark-adapted peels were illuminated with 10 μmol m^{-2} s^{-1} blue light superimposed on 50 μmol m^{-2} s^{-1} red light (n = 100; ±SEM). An unpaired Student's t test determined statistical significance between the indicated comparisons (*$p < 0.001$; n.s., not significant).

(E and F) Stomatal aperture (E) and guard cell starch content (F) in epidermal peels treated for 1 hr and 3 hr with or without 10 μM fusicoccin (Fc) in opening buffer containing 50 mM KCl. 1 hr dark-adapted peels were illuminated with 10 μmol m^{-2} s^{-1} blue light superimposed on 50 μmol m^{-2} s^{-1} red light (n = 80; ±SEM). An unpaired Student's t test determined statistical significance between the indicated comparisons (*$p < 0.001$; n.s., not significant).

See also Figure S3.

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AUTHOR CONTRIBUTIONS

D.S. conceived the project. D.S. and D.H. designed the experiments. D.H., S.F., D.P., J.S.A.M., M.T., and A.N. performed the experiments and analyzed the data. D.S., D.H., and T.L. interpreted the data. N.L. contributed new reagents/analytic tools. D.S. and D.H. wrote the paper.

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Conclusion and Outlook

Osmotic stress induces starch degradation in plants

Our results show that in Arabidopsis plants, transitory starch is degraded not only during the night, but also under osmotic stress conditions. This effect could be observed both during acute stress (chapter 1) and prolonged exposure to a milder stress (chapter 2). Mutants unable to degrade starch during osmotic stress were more sensitive to stress, accumulated less compatible solutes (chapter 1 and 2), exhibited reduced root growth and water uptake (chapter 1) and showed more lipid peroxidation (chapter 2). Therefore, it appears that stress-induced starch degradation increases the plant's stress tolerance and fitness. By analysing and comparing studies from many different species, we could further show that stress-induced starch degradation occurs in many different plant species, including algae and mosses (chapter 5). This indicates that the remobilisation of starch represents an old and conserved stress response in the plant kingdom.

Stress-induced starch degradation relies on different set of enzymes than nocturnal starch degradation

We analysed the mechanism underlying stress-induced starch degradation in detail, and found that AMY3 and BAM1 are key enzymes in this process. The loss of either enzyme reduces stress-induced starch degradation (chapter 1). In contrast nocturnal starch degradation was unaffected in either single mutant as well as in the *amy3bam1* double mutant. This contrasts with BAM3: while stress-induced starch degradation is not impaired in the *bam3* mutant (chapter 1 and 2), nocturnal starch degradation is impaired by the loss of BAM3 (Fulton et al., 2008). This subfunctionalisation of different enzymes indicates that they have been adapted for their different functions. Indeed, Monroe et al., (2014) found that BAM1 and BAM3 are adapted to high and low temperature which is consistent with their proposed function during the day and the night, respectively.

Additional enzymes may participate in stress-induced starch degradation

Our work clearly shows that AMY3 and BAM1 are essential for stress-induced starch degradation. However, it is likely that they are not the only enzymes involved. Previous research indicated that PHS1 does contribute to drought stress resistance (Zeeman et al., 2004) and in line with these results we found *PHS1* expression to be induced by osmotic stress (Addendum to chapter 1). To further investigate the role of PHS1 during

water stress, *phs1* mutants were obtained and *amy3/phs1*, *bam1/phs1* and *amy3/bam1/phs1* multiple mutant were generated. Analysis of this mutant set will establish the function of PHS1 during water stress as well as its interaction with AMY3 and BAM1.

None of these three enzymes cleave the α-1,6-branchpoints of amylopectin, and therefore the AMY3, BAM1 and PHS1 alone are not able to degrade starch completely. Consequently, at least one debranching enzyme must also participate in stress-induced starch degradation. We found that the expression of two genes encoding for debranching enzymes, *ISA3* and *LDA*, are induced by osmotic stress (Addendum to chapter 1), making it likely that both enzymes participate in stress-induced starch degradation. To clearly establish their role in stress-induced starch degradation, as well as the relative contribution of each enzyme mutants lacking either ISA3 or LDA or both enzmyes should be subjected to osmotic stress. A potential complication for such experiment is the starch excess of the *isa3* mutant. As shown in chapter 4 the amount of starch can influence the response to osmotic stress. To account for this problem, the *bam3* mutant should be included as a control, as the mutant does show a starch excess but is not impaired in starch degradation.

In addition to hydrolytic enzymes, nocturnal starch degradation also depends on glucan phosphorylation – mediated by the two kinases GWD and PWD – and dephosphorylation – mediated by the phosphatases SEX4 and LSF2. While we did not investigate the role of reversible phosphorylation during osmotic stress, the severe starch excess of *gwd* and *sex4* mutants indicates a central role in this process, making it likely that it also plays a role in stress-induced starch degradation.

Furthermore, nocturnal starch degradation relies on several non-catalytic proteins such as BAM4 and LSF1. Although the physiological role of these proteins has so far not been established, it is possible that one or several proteins participate in stress-induced starch degradation.

To determine if any of these proteins participate in stress responses, their expression in response to osmotic stress and ABA should be analysed which would provide a list of candidate genes. Subsequently, starch metabolism during stress should be analysed in these mutants to verify the function of the corresponding protein during osmotic stress. This would allow us to assemble the complete pathway of stress induced starch degradation.

Transcriptional regulation of BAM1 *expression merits further investigated*

We showed that *BAM1* is induced by both osmotic stress and the hormone ABA. Furthermore, our results indicated that the ABA-mediated induction depends on the

canonical ABA signalling pathway (chapter 1). We also found ABA responsive elements in the promoter of *BAM1* and its orthologs, suggesting that BAM1 may be a direct target of the AREB/ABF transcription factors. However, we did not directly demonstrate this interaction, leaving open the possibility that BAM1 is activated indirectly by other targets of AREB/ABFs. To further investigate the activation of BAM1 in response to ABA three complementary techniques are available: The binding of transcription factors to DNA can be analysed *in vitro* by electrophoretic mobility shift assays (EMSA) (Hellman and Fried, 2007), and *in vivo* using chromatin immunoprecipitation (ChIP) (Nelson et al., 2006). Furthermore, the activation of gene expression can be investigated *in vivo* by transient expression in Arabidopsis mesophyll protoplasts (TEAMP) (Yoo et al., 2007). EMSA relies on the fact that during electrophoresis protein-DNA complexes migrate at a lower speed than free DNA. Therefore, if the AREB/ABF transcription factors are able to bind the *BAM1* promoter directly, it is expected that the *BAM1* promoter will migrate more slowly in the presence of these proteins. If the wild type *BAM1* promoter is indeed able to interact with the transcription factors the exact sequence(s) necessary for binding can be determined by targeted mutagenesis. The two conserved motifs (ABRE and CE3-like) in the *BAM1* promoter we identified (chapter 1) are obvious targets for this analysis. ChIP allows the analysis of the *in vivo* interactions between proteins and DNA. In a first step proteins and DNA are crosslinked using formaldehyde, subsequently the DNA is sheered, and the protein of interest (in our case the AREB/ABF transcription factors) are precipitated using a specific antibody. If the BAM1 promoter is enriched in the immunoprecipitated material, this would indicate that the AREB/ABF transcription factors did interact with the BAM1 promoter *in vivo*.

TEAMP assays measure the ability of a transcription to activate the expression of a reporter gene from a given promoter. To test if the ABRE/ABF transcription factors are able to activate the *BAM1* promoter, plasmids expressing a reporter gene under the control of the *BAM1* promoter are transfected either alone or with plasmids containing the transcription factors under a constitutive promoter. If the transcription factors are able to bind the *BAM1* promoter and increase the transcription of the reporter gene, it will be possible to identify the responsible motifs using constructs with mutated versions of the promoter.

Taken together, the results of these experiments will provide a conclusive answer to the question as to whether BAM1 is a direct target of the ABA signalling pathway, and will validate the function the motifs identified *in silico*.

The circadian clock affects stress-induced starch degradation

While osmotic stress clearly induces starch degradation at noon (chapter 1), this induction was not observed later during the day (chapter 4). Preliminary results indicate that this difference is due to the circadian clock protein TOC1. In *toc1* mutants starch degradation still occurred towards the end of the day. TOC1 has previously been proposed as an inhibitor of ABA responses (Legnaioli et al., 2009), and it is possible that it inhibits stress-induced starch degradation by repressing ABA signalling. Further work will be necessary to unambiguously establish the role of the circadian clock in stress-induced starch degradation. As a first step it be would necessary to investigate the strength of ABA-induced *BAM1* expression in Col-0 over the course of a day, to see if ABA-dependent *BAM1* induction is indeed gated by the circadian clock. Furthermore, a complete analysis of the *toc1* mutant and its responses to ABA and osmotic stress needs to be conducted as described in chapter 4 (Page 99). These results would help us to understand how the circadian clock affects starch metabolism under stress conditions. In addition one may also investigate the influence of the circadian clock on responses to long term stress. As shown in chapter 2, BAM1 also degrades starch in response to prolonged moderate osmotic stress. As this stress extended over several day-night cycles it would be interesting to investigate the precise timing of this degradation over the course of the day. If prolonged stress elicits a similar response as acute stress, it is expected that the stress-induced starch degradation occurs primarily during the middle of the day and is reduced towards the end of the day. Conversely, it is also possible that long-term stress results in different responses and starch degradation is induced throughout the day.

Analysis of stress-induced starch degradation in other species

Our results highlight the importance of stress-induced starch degradation in Arabidopsis, and the existing literature supports the hypothesis that remobilisation of starch is a conserved stress response (chapter 5). Furthermore, a phylogenetic analysis of BAM1 revealed that the protein is conserved amongst all flowering plants (chapter 6). As the ABA responsive elements were found in the promoters of most eudicots species analysed (chapter 1), it is likely that BAM1 is also involved in stress-induced starch degradation in these plants. However, we did not analyse the stress responses of other plants species to experimentally confirm this theory. In recent years, great advances have been made in developing tools and resources for non-Arabidopsis plants, important resources such as “omics”, T-DNA libraries and efficient transformation techniques are now available for many plant species. Using these resources it will be possible to analyse the stress responses in several crop species

such as canola (*Brassica rapa*), a close relative of Arabidopsis, tomato (*Solanum lycopersicum*), a more distantly related eudicot, and rice (*Oryza sativa*) a monocot. In a first step, the changes in transcription and starch metabolism during osmotic stress or ABA treatment in these organisms should be surveyed. Depending on the results, mutants unable to synthesise ABA, or lacking the orthologs(s) of BAM1 and AMY3 could then be analysed in a second step. In some species, already published mutants could be used, such as the ABA deficient *sitiens* or *flacca* mutants in tomato (Taylor et al., 1988), but other mutants need to be created using gene silencing/genome editing. Careful investigation of these mutants would allow to determine if stress-induced starch degradation in crop plants relies on the same enzymes and signalling pathways as in Arabidopsis.

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